University of Alberta

Development of a Nanoparticle-based Vector for Gene Delivery

by

Yuan Huang

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Abstract

The goal of the project was to develop a nanoparticle-based carrier for possible drug targeting and gene delivery.

A two-step desolvation process was used to synthesize nanoparticles from gelatin A and B. The important synthesize parameters were investigated. The DNA binding capacity of gelatin A and B nanoparticles was conformed by agarose gel electrophoresis. COS-1, 143B, HELA and 293 cells were used for the gene transfection studies *in vitro*. Lac Z was used as a reporter gene for detection of transfection.

Controlling the experimental conditions, nanoparticles with defined size ranges and narrow size distribution can be synthesized. Gelatin A nanoparticles have a higher binding capacity compared to gelatin B nanoparticles. Therefore gelatin A nanoparticles were selected as vector for gene transfection studies. Gelatin A nanoparticles showed the ability to deliver the Lac Z gene to COS-1 and 143B cells.

Dedicated to with love:

My husband: Zhurang Zhao

My parents: Xiaochun Huang and Wanfen Li

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List of Abbreviations

BSE	bovine spongiform encephalopathy
DLS	dynamic light-scattering
DOPE	dioleoylphosphatidylethanolamine
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
GA	glutaraldehyde
GPC	gel permeation chromatography
GRF	growth hormone releasing factor
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid
HPLC	high performance liquid chromatography
pI	isoelectric point
kb	kilobase
MPS	mononuclear phagocytic system
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NP	nanoparticle
PBS	phosphate buffered saline
PEI	polyethylenimine

PEG	polyethylene glycol
PLL	poly(L-lysine)
PLGA	poly(D,L-lactide- *co*-glycolide)
RES	reticuloendothelial system
SCID	severe combined immune deficiency
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	scanning-electron-microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TSE	transmissible spongiform encephalopathy,
X-gal	5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside
ZCI	zyderm collagen implant

Chapter 1

Introduction

1.1. Overview of nanoparticles and gene delivery

In 2000, Human Genome Project leaders announced the completion of a "working draft" DNA sequence of the human genome. Then in 2003, the Human Genome Project finished with the completion of the human genetic sequence (http://www.ornl.gov/TechResources/Human_Genome/home.html). Today gene therapy is considered as a very promising technology because of the advancements in the Human Genome Project.

Nano-technology is another frontier area of science. It contributes many areas of society, which involves multidisciplinary scientific approaches in engineering, chemistry, physics, medicine and pharmaceutical sciences.

Nanoparticles are a promising gene delivery system for medical applications. They may combine both frontier areas, gene therapy and nano-technology, together. In this project, gelatin was selected as biomaterial to synthesize nanoparticles.

1.2. Gelatin

Gelatin is a protein that is widely used in applications ranging from pharmaceutical and medical use to food sciences, cosmetics and photography. Gelatin is non-toxic, of low immunogenicity and biocompatible biomaterial (Rose, 1990; Esposito *et al.*, 1996; Brine *et al.*, 1992). The history of gelatin can be traced back to at least hundreds of years ago. As early as in the mid of 17th century, the earliest commercial product of gelatin was manufactured in Holland. About 1700, the manufacture of

gelatin began in England. In the late 18th century, the production in France began. In 1808, gelatin was first made in North America (http://www.gmap-gelatin.com). Now it is produced and applied worldwide in pharmacy, health, food, cosmetics, and photography applications.

1.2.1. Collagen

Gelatin is made from collagen. Collagen is the most abundant single protein in most vertebrates. Collagen is the matrix material in the bones; it forms the major portion of tendons; and it is an important constituent of skin. Collagen provides structure to our bodies, protecting and supporting the soft tissues and connecting them with the skeleton.

Until now there are at least 19 types of collagen identified (Timpl, 1984). Type I collagen is predominant in higher order animals especially in the skin, tendon, and bone where extreme forces are transmitted. The basic unit of collagen is the tropocollagen molecule, a triple helix of three polypeptide chains. In Type I collagen, there are two α1(I) chains and one α2(I) chain (Kucharz, 1992). Each of these three chains has a repetitive sequence of three amino acid residues, Gly-X-Pro or Gly-X-Hypro, where X is some other amino acid (see Figure 1.1 a) (Campbell, 1995; Friess, 1998). Each strand is a left-handed helix twisted on itself with 3.3 residues every turn and a pitch of 0.87 nm (Kucharz, 1992; Brodsky *et al.*, 1988). Three strands are held together by hydrogen bonds to form a larger right-handed triple helix (see Figure 1.1 b) (Campbell, 1995; Friess, 1998). This rod-shaped triple-helix has a length of 300 nm, a diameter of 1.5 nm and a mean molecular weight of about 300 kDa (Nimni and

Harkness, 1988). These individual tropocollagen molecules align side by side in a staggered fashion to form the fiber (see Figure 1.1 c) (Campbell, 1995; Friess, 1998).

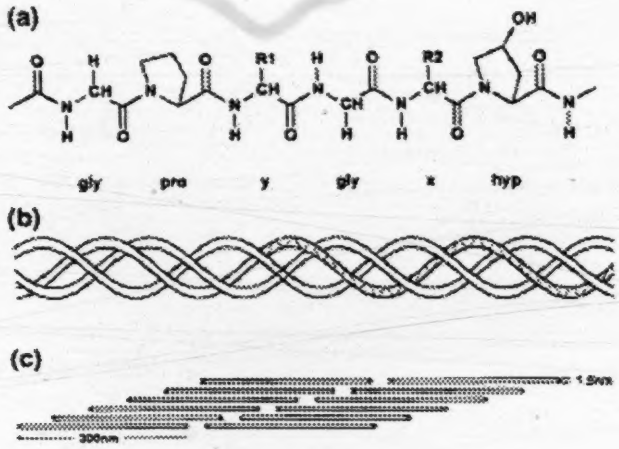

Figure 1.1 Chemical structure of collagen type I. (a) Primary amino acid sequence, (b) secondary left handed helix and tertiary right handed triple-helix structure and (c) staggered quaternary structure (Friess, 1998).

Type I, II, III, V and XI collagens are made up by the continuous triple-helical structure (Timpl, 1984). In type IV collagen, the triple-helical regions are interrupted with large non-helical structures and the short non-helical peptide interruption. Type IX, XI, XII and XIV collagens have small chains that have non-helical regions. Type VI collagen is microfibrillar collagen and type VII is anchoring fibril collagen (Samuel *et al.*, 1998).

1.2.2. The manufacture of gelatin:

Gelatin is made from the collagen of animal skins/hides, bones and tendons and connective tissues (Bello and Vinograd, 1958; Eastoe, 1955). The collagen protein is extracted from the cattle bones, calf hides or pigskins and is manufactured into gelatin. Because of various collagen sources, the manufacturing techniques are different. However, the principle of preparing gelatin is almost the same. The composition of various gelatins depends on the source of collagen and the hydrolytic treatment used. Usually there are several processes in the manufacturing process, including pretreatment, extraction, cleaning, concentration, and drying.

In the pretreatment process, the materials are washed and cut. The non-collagenous materials like fat and minerals are removed as much as possible. Then two different kinds of pretreatment: alkaline processing or acid processing, are applied. Gelatin A is produced by acid processing, while gelatin B is produced by the alkaline method. Pigskin is usually used as raw material in the acid process. This is due to the fact that the collagen connective tissues from pigskin are not very heavily interconnected and the collagen molecule comes from young animals. A one-day treatment in an acid solution (such as hydrochloric acid and sulfuric acid) is usually sufficient to extract the collagen.

The common raw materials in the alkaline process are beef hides or bones. The collagen molecules in cattle skin or bones are heavily cross-linked; therefore the pretreatment with alkaline solution (like lime) takes several weeks.

After the pretreatment process, the material is ready for extraction with hot water. Gelatin is fractionally extracted by heating. Earlier extractions made at lower

temperature have higher molecular weight, higher viscosity, and higher gel strength. Later extractions made at higher temperature have inverse properties. To get the final product, the extract will be filtered, centrifuged, and dried (Salo, 1949; http://www.gelatine.org/index.html; http://www.gelatin-gmia.com/)

1.2.3. Chemical composition and structure of gelatin:

Commercial gelatins are heterogeneous protein mixtures of polypeptide chains. As with all proteins, the sequence and type of the amino acids in the chain determines the functionality of the chain. Gelatin is composed of 18 different amino acids. Characteristic features of gelatin are the high content of the amino acids glycine, proline and hydroxyproline (see Figure 1.2) (http://www.gelatin.com). The absence of tryptophan and aromatic acid residues has been underlined (Bender *et al.*, 1953; Loofbourow *et al.*, 1949).

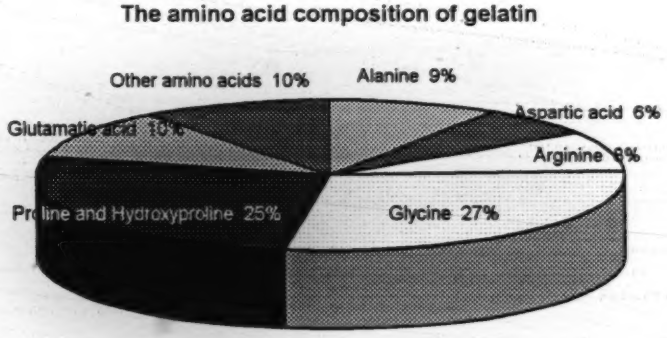

Figure 1.2 The amino acid composition of gelatin (modified from PB Leiner, http://www.gelatin.com)

1.2.4. Properties and Characteristics of Gelatin

Because of the different processes of hydrolysis, gelatin A and B differ in their isoelectric point. Gelatin A has an isoelectric point from 7 to 9 and the isoelectric point of gelatin B is in the range of 4.8 to 5.1 (Kobayashi, 1996). As the pH increases gelatin proteins in a solution become more negative because the carboxyl groups will be de-protonated. Conversely, gelatin is more positively charged at lower pH values because of the protonation of amino groups. The molecular charge of gelatin is a critical characteristic for its application. At different pH values the different gelatin types will exhibit different charges and this might be used for drug loading and DNA condensation.

The molecular weight range of gelatin molecules is very large, from a few thousand up to several hundred thousand daltons. The molecular weight distribution of gelatin has effects on the physical properties of gelatin and particularly affects viscosity and gel strength values. The strength of a gelatin product is described by the bloom value. The bloom value is a measurement of the strength of a gelatin gel by a Bloom Gleometer (a device that make this determination under standardized conditions). It measures the weight in grams that is required to depress a standard plunger 4 millimeters into a gel at 10° C. For example, if this procedure requires 200 grams, the gelatin is a 200 bloom gelatin (USP 24 NF 19, 2000; http://www.milligan1868.com/gelatins.html). Gelatin is a water-soluble protein. The rate of solubility is affected by factors such as temperature, concentration and particle size. Gelatin is insoluble in alcohol and most other organic solvents. The viscosity of gelatin varies with the type of gelatin, the molecular weight distribution of the gelatin

molecules, concentration and temperature. The viscosity of gelatin solutions increases with increasing concentration and with decreasing temperature (http://www.gelatin.com).

1.2.5. Pharmaceutical and medical applications of collagen

Collagen is a traditionally used biomaterial that is widely used in pharmaceutical and medical sciences.

Collagen can be produced in different formulations, like tubes for vessel prostheses, sponges for wound dressings, powders for cosmetic in creams, and injectable solutions used in skin cosmetic defects (Ochiya *et al.*, 2001). Such systems are also investigated their potential use as drug delivery system in a number of applications such as tumor treatment (Davidson *et al.*, 1995; Ning *et al.*, 1996), ophthalmology (Rubin *et al.*, 1973; Poland and Kaufman, 1988), burn dressing (Papp and Harma, 2003), and tissue engineering (Royce *et al.*, 1995; Kenley *et al.*, 1993). Furthermore, collagen was explored as carrier for gene delivery. Fang *et al.* used collagen sponges as an implantable carrier for plasmid DNA (Fang *et al.*, 1996). The nonviral vector loaded collagen sponges were tested for sustained gene delivery *in vitro* and *in vivo* (Scherer *et al.*, 2002). They compared lipoplex- and polyplex- (polyplexes-cationic polymer/DNA complexes; lipoplexes -cationic lipid/DNA complexes) loaded collagen sponges with naked DNA-loaded sponges. Lipoplex- and polyplex-loaded collagen sponges are greater in mediating sustained gene delivery *in vitro* and local transfection *in vivo* as compared to naked DNA-loaded sponges.

Tyrone *et al.* used collagen as carrier for gene therapy in wound healing (Tyrone *et al.*, 2000). This study tested the wound healing effects of topically applied platelet-derived growth factor (PDGF) DNA embedded within a collagen matrix. PDGF DNA gene therapy is effective at accelerating wound healing in ischemic dermal ulcers. Atelocollagen, a decomposition product of type I collagen extracted from the dermis of cattle, has shown its potential as delivery system, especially for gene delivery. Plasmid DNA was embedded in the Minipellet that was made by atelocollagen. The Minipellet based delivery had longer release of DNA than that of plasmid DNA alone *in vivo* because Minipellet protected DNA from biodegradation (Kohmura *et al.*, 1999; Ochiya *et al.*, 1999; Ochiya *et al.*, 2001).

1.2.6. Pharmaceutical and medical applications of gelatin

In the pharmaceutical industry, the most common usage of gelatin is to make hard and soft gelatin capsules. A lot of research has been done to investigate the use of gelatin as a plasma substitute. Gelatin has already been served as plasma extender in the clinic (Lundsgaard-Hansen and Tschirren, 1978; Broghammer, 1969; Kief, 1969; Mishler, 1984).

In recent years, there is rapid progress in artificial skin engineering using gelatin as the raw material. For example, gelatin was used in wound healing. Grosslinked gelatin hydrogel was developed into a wound-dressing membrane *in vivo* by a rat model (Chang *et al.*, 2003).

Gelatin is also valuable in research of bone regeneration: TGF beta 1-gelatin hydrogel is surgical tool for skull defect repair and skull base reconstruction (Hong *et al*,

2000). It is not surprising that gelatin is applied in preparing injectable biomaterials for bone surgery as well (Zahraoni and Sharrock, 1999). Gelatin is also used as a raw material in prosthetic heart valves for intravascular application (Hoffman *et al.*, 1993). Furthermore, Gelatin has been used as a drug carrier and is noted for its biocompatibility and absence of toxicity (Rose, 1990; Esposito *et al.*, 1996). Its properties are usually described as bioabsorbable, non-toxic, and minimally immunogenic (Brine *et al.*, 1992).

1.2.7. Immunogenicity of gelatin and collagen

There are various applications of gelatin and collagen. Therefore, the research on gelatin's and collagen's immunogenicity has progressed quickly. In the 1980's, Delustro *et al.* published a research paper to compare the biologic and immunologic response of different medical device made from dermal collagen. They examined 7 collagen materials for soft tissue augmentation in a subcutaneous guinea pig model, including ZCI®, GAX collagen, Atelocollagen, Gelfoam®, Avitene®, Collastat®, and reconstituted, intact fibrillar collagen from bovine skin (see Table 1.1).

Table 1.1 Different types of collagen materials (DeLustro *et al.*, 1986; http://www.rxmed.com/)

Abbreviations	Full names	Properties
ZCI	Zyderm® Collagen Implant	A pepsin-solubilized injectable fibrillar collagen that has been applied in soft tissue augmentation in the correction of contour deformities.
GAX collagen	Glutaraldehyde cross-linked collagen	An injectable material is similar to ZCI but it is lightly crosslinked by glutaraldehyde. It was also designed for soft tissue augmentation.
Atelocollagen	Koken Atelocollagen	An injectable collagen is applied in soft tissue augmentation.
Gelfoam	Gelfoam® Gelatin Powder	It is intended for application to bleeding surfaces as a hemostatic agent.
Avitene	Avitene® Microfibrillar Collagen Hemostat	An absorbable, topical, hemostatic agent, and is applied to control bleeding in some surgical situations.
Collastat	Collastat® Collagen Hemostat	An absorbable collagen sponge is used as a hemostatic agent.
	Intact collagen	Reconstituted, intact fibrillar collagen from bovine skin.

After three injections, serum antibodies were measured using ELISA to test the immunogenicity. They found Avitene was the most immunogenic, while GAX collagen demonstrated the greatest persistence and minimal immunogenicity, and ZCI was the least immunogenic of the collagen materials examined (DeLustro *et al.*, 1986). In 1981 and 1985, Zyderm and Zyplast collagen implants, two injectable suspensions of purified bovine dermal collagen, were commercially used in the USA. More than 750 000 patients have been treated. Only a small percentage of treated patients had adverse reactions to bovine collagen implants. The most prevalent adverse reaction was localized hypersensitivity at treatment sites (Keefe *et al.*, 1992). Since the end of 1990's, in Japan there are many reports about anaphylactic reactions to measles, mumps, and rubella vaccines that include gelatin as a stabilizer. It was found that most of these reactions are caused by the bovine gelatin included in these vaccines. Gelatin mainly includes denatured type I collagen, which consists of α1 and α2 chains. In gelatin allergy denatured bovine type I collagen is a major allergen and IgE-binding sites exist in the α2 chain of type I collagen (Miyazawa *et al.*, 1999; Sakaguchi and Inouye, 2000; Sakaguchi *et al.*, 2000; Sakaguchi *et al.*, 1999; Kumagai *et al.*, 1997).

Before a biomaterial can be applied *in vivo*, immunogenicity of this biomaterial must be cleared. From the literature review, we can see that gelatin and collagen are widely used biomaterials. Many experiments about both biomaterials have been performed in different animal models. Products from these biomaterials are already clinically used. Although gelatin and collagen exhibit a low immunogenicity compared with other

materials, it is still very meaningful to develop more purified gelatin and collagen to obtain no-, or low, immunogenicity.

1.2.8. Gelatin and Bovine spongiform encephalopathy (BSE)

Cattle are the main source for collagen and gelatin. Because of BSE or TSE (transmissible spongiform encephalopathy), the safety of gelatin becomes an issue. But the risk of contamination is very low because of the harsh condition during manufacturing process. As described in 1.2.2, beef hides or bones are usually processed by alkali treatment and it takes several weeks. After the alkali treatment, the risk is obviously reduced.

The manufacturers can also carefully select the origin of cattle. This will control the risk of infection.

1.3. Nanoparticles

Nanoparticles are solid colloidal particles, which size range from 10-1000 nm in diameter (Kreuter, 1994). Nanoparticles were first developed around 1970. They were initially devised as carriers for vaccines and anticancer drugs (Couvreur *et al.*, 1978). Now nanoparticles are used as drug targeting devices for therapeutic moieties. Drugs can be dissolved, entrapped or encapsulated into the nanoparticles, or attached to their surface. This new system offers numerous advantages compared to the use of conventional dosage forms, which include improved efficiency, reduced toxicity, overcoming drug resistance and improved patient compliance.

1.3.1. Characteristics of nanoparticles

Nanoparticles are characterized by the following parameters:

- They are small enough to get into the target cell and tissues (particle size ranges from 10 to 1000 nm in diameter).
- They can deliver drugs or DNA to the target cell.
- The surface of nanoparticles can be modified to reduce the uptake by the reticuloendothelial system (RES) and to protect the drugs or DNA from endosome-lysysome processing following endocytosis.
- Nanoparticles can be coated by some materials that will increase the cellular uptake of particles by specific organs (Araujo *et al.*, 1999)
- Specific antibodies can be attached to nanoparticles. This can be used to target specific cells.

Because of these special characteristics, nanoparticles are compelling and promising as a drug targeting and gene delivery system.

1.3.2. Nanoparticles and the Reticuloendothelial System

After nanoparticles are administered by intravenous injection, the reticuloendothelial system (RES), also called the mononuclear phagocytic system (MPS), takes up nanoparticles. The intravenously injected colloids are mostly distributed into the RES organs: liver (60-90%), spleen (2-10%), lungs (3-20% and more), and bone marrow (>1%) (Kreuter, 1983; Illum *et al.*, 1984; Kreuter, 1985; Kreuter, 1994).

A lot of research focused on the development of methods to reduce the uptake of the nanoparticles by the cells of the RES (Illum *et al.*, 1987). Coating the particles is an

efficient way to decrease the RES uptake. For example, poly(methyl methacrylate) nanoparticles were coated with poloxamine 908 and polysorbate 80. At a 0.1% concentration of poloxamine 908, the liver concentration of nanoparticles decreased from about 75% of the administered dose to 13%. At the same time, the blood concentration and the concentration in other organs and tissues were increased. The decrease of the nanoparticle concentration in the liver and the increase in the blood and the other organs became important at above 0.5% for polysorbate 80 coated particles (Araujo *et al.*, 1999).

Today it is well established by different *in vivo* studies using liposomes and nanoparticles that protective coatings of colloidal drug carriers can inhibit the RES uptake. The liposome system with such properties is called Stealth liposomes. These are classical liposomes that contain a layer of monosialoganglioside or polyethylene glycol-distearoyl phosphatidyl ethanolamine shielding to reduce interactions with serum components and the RES (Allen and Hansen 1991; Allen *et al.*, 1992). Consequently, such carriers have a much longer circulation time than non-stealth liposomes. Reduced macrophage uptake of nanoparticles made of poly (lactic acid), poly(ethylene oxide) diblock and triblock copolymers (PEO) have been developed as injectable nanoparticles. This approach was chosen to circumvent the lack of stability of PEO coatings obtained by simple surface adsorption. The stability of PEO coatings has been shown to be of great importance for ensuring efficient nanoparticle protection against the mononuclear phagocyte system (De Jaeghere *et al.*, 2000).

1.3.3. Applications of Nanoparticle

Delivery systems in the nanometer area have a high potential for drug delivery. Currently, nanoparticles have been investigated in various applications including anti-cancer, anti-infection, delivery of peptide drugs, gene delivery and so on (see Table 1.2).

Table 1.2 Applications of nanoparticles

	Applications	Examples
Nanoparticles	Anti-cancer therapy	• Doxorubicin • Mitoxantrone
	Anti-infectious therapy	• Amikacin • Ampicillin • Primaquine
	Peptide drug delivery	• Insulin • Growth hormone releasing factor

Anti-cancer therapy

Nanoparticles have potential use as delivery systems for anti-cancer drugs at specific target tumor sites. Previous studies have shown that nanoparticles tend to accumulate in some tumors after intravenous injection (Gipps et al, 1986; Beck *et al.*, 1993; Keuter 1994). This new dosage form may improve the efficiency and reduce the toxicity of many drugs. For example, doxorubicin has severe cardiotoxicity but when

associated with nanoparticles, the heart accumulation was decreased; thus decreasing the toxicity in the heart. Nanoparticles also obviously decreased the acute toxicity of doxorubicin compared with free doxorubicin in mice after intravenous injection (Couvreur, 1982; Couvreur, 1986). Another study showed that mice with lymphoid leukemia injected with doxorubicin bound to nanoparticles had twice the survival rate (%) than those with free doxorubicin. It was also found that doxorubicin with nanoparticles could be administered at doses that would have otherwise been toxic for free doxorubicin (Couvreur and Vauthier, 1991).

Another example of the potential of nanoparticular systems is that Poloxamine 1508-coated nanoparticles increase the delivery of anticancer drugs such as mitoxantrone to solid tumors (Beck *et al.*, 1993; Reszka *et al.*, 1997).

Anti-infectious therapy

Nanoparticles have great promise for delivering anti-infectious drugs and have been applied in the treatment of infections.

A lot of research has been undertaken using nanoparticles as carriers for antibiotic delivery systems. Many anti-infective drugs were bound to nanoparticles and their *in vitro* release was investigated such as amikacin (Losa *et al.*, 1991), ampicillin (Baszkin *et al.*, 1987; Youssef *et al.*, 1988; Fattal *et al.*, 1989), and primaquine (Labhasetwar and Dorle, 1990). From all of these drugs, ampicillin was studied most. For example, the effectiveness of ampicillin bound to nanoparticles was increased compared with free ampicillin in mice when ampicillin was used in treatment of Listeria monocytogenes (Youssef *et al.*, 1988) and salmonellosis (Fattal *et al.*, 1989).

Furthermore, nanoparticles are promising drug carriers in AIDS therapy because they are easily taken up by cells of RES (Löbenberg and Kreuter, 1996). The macrophages of the RES are an important reservoir (Gendelman *et al.*, 1989) for HIV and play a detrimental role in the progression of AIDS (Orenstein *et al.*, 1997). Nanoparticles also could improve the delivery of antiviral agents to the mononuclear phagocyte system *in vivo* and enhance the activities of drugs for the treatment of HIV infection and AIDS (Bender *et al.*, 1996).

Finally, nanoparticles are not limited for the treatment of bacteria and viruses, but also have been explored in the use of antiparasitic and antifungal drugs (Couvreur and Vauthier, 1991).

Peptide drug delivery

In the last 20 years, peptide drugs have become more and more important. Their instability in the bloodstream is one of the key problems with these molecules. The half-life of many peptides is short and peptides are vulnerable to decomposition by enzymes, therefore, they have to be protected to achieve an efficient delivery (Sehgal, 2003). Subsequently, the research on the delivery of peptide drugs is highly demanding.

In a study, insulin was encapsulated into nanoparticles (Damgé *et al.*, 1988). The insulin nanocapsules were subcutaneously injected to rats. A significant prolongation of blood glucose level reduction was achieved compared with free insulin. After peroral administration, it was found that the nanoparticles were able to protect insulin from degradation by gastric fluids.

The binding of growth hormone releasing factor (GRF) to nanoparticles was studied by Grangier *et al.* (Grangier *et al.*, 1991) and Gautier *et al.* (Gautier *et al.*, 1992). Compared with free GRF, GRF with nanoparticles can maintain longer constant plasma levels of GRF after subcutaneous administration. This result showed that nanoparticles might be used as sustained release systems for peptide drugs.

The above examples demonstrate that nanoparticles are the promising carriers for peptide drugs.

1.4. Nanoparticles in gene delivery

1.4.1. An overview of gene delivery

Gene therapy is a new and promising area in pharmaceutical and medical sciences because it has the potential to treat diseases at the genetic level. The definition of human gene therapy is: "the transfer of nucleic acids to somatic cells of a patient which results in a therapeutic effect, by ether correcting genetic defects or by overexpressing proteins that are therapeutically useful" (Rubanyi, 2001).

The basic idea of gene therapy is not complicated – transfer the healthy DNA to disease-affected cells. It is feasible to treat diseases of genetic origin or correct genetic deficiencies by administering healthy copies of the mutated genes. This might become possible through developments in molecular biology. The completion of a working draft of the human genome project is one milestone in this approach (Macilwain, 2000). Early gene therapy has shown its potential in the treatment of many severe diseases (i.e., hypertension, asthma, Parkinson's diseases, motor neuron disease, multiple sclerosis) (Dalgleish, 1997), different forms of cancers (Felgner *et*

al., 1995; Chong and Vile, 1997; Niculescu-Duvaz *et al.*, 1998; Rolland and Felgner, 1998; Gomez-Navarro *et al.*, 1999), AIDS (Garnett, 1999) and other diseases, including metabolic disorders (e.g., diabetes and hypercholesterolemia) (Rolland and Felgner, 1998), heart disease (Smith *et al.*, 1995), vascular disease (Svensson and Schwartz, 1998) and kidney disease (Imai and Isaka, 2000). The first clinical trial of gene therapy was done in 1990 in children with adenosine deaminase deficiency (Dube and Cournoyer, 1995). Since then, over 600 worldwide clinical trials in gene therapy with about 3500 involved patients demonstrate the rapid progress in gene therapy (http://www.wiley.com/legacy/wileychi/genmed/clinical/).

1.4.2. Viral vectors and nonviral vectors

Gene-therapy vectors can be placed in two broad categories: viral vectors and nonviral vectors (Anwer *et al.*, 2000; Salyapongse *et al.*, 1999; Ledley, 1995). One is the virus-based method, containing retroviruses, adenoviruses and adeno-associated viruses.

The other one is the nonviral vector. The definition of nonviral vectors is broad. The transfection strategies include the injection of naked DNA, gene guns (Hui and Chia, 1997), electrical pulses (Oshima *et al.*, 1998), ultrasound (Lauer *et al.*, 1997), calcium phosphate precipitation and synthetic vectors for gene therapy. The majority of nonviral vectors are synthetic systems (Luo and Saltzman, 2000).

Each viral and *non-viral* approach has its own advantages and disadvantages. Until now viral vectors are the most effective ways of DNA delivery. The vectors have a high efficiency in DNA delivery (i.e., fraction of DNA getting into the nucleus) and

gene expression (i.e., fraction of nuclear DNA that undergoes transcription). Such recombinant viral vectors are applied in about 75% of late clinical protocols in gene therapy (Luo and Saltzman, 2000). However, the drawbacks of viral vectors are apparent. The major concerns of viral vectors are safety reasons. One recent example of the high risk in using viral vectors for gene therapy was shown in a French young boy suffering from severe combined immune deficiency (SCID). The child was treated by gene therapy and developed a form of cancer obviously caused by the treatment that cured his SCID (Check, 2002). Toxic immunological reactions are another major disadvantage of viral vectors. An example was that a patient with a rare metabolic liver disorder died due to the adenovirus vector he received during a gene-therapy treatment (Marshall, 1999). In this case, the adenovirus invaded not only the intended target (liver), but also other organs triggering an activation of innate immunity that led to fatal multiple organ failure. The viral vectors still have other limitations, including the uncertain long-term effect of the integrated transgene and the virus in the host individual, low virus titer, restricted targeting of defined cell type specificity, limited size of incorporated DNA, manufacturing and packing problems, recombination, and high cost (Boyce, 2001; Crystal, 1995; Tripathy *et al.*, 1996).

On the other hand, the most significant drawback of the *non-viral* system is inefficiency gene delivery system compared to a viral system. But the *non-viral* vector has its own advantages, including easily controlling their composition for simple analysis and production, able to large-scale production, flexibility in the size of the DNA to be delivered (Thomas and Klibanov, 2003; de Jong, *et al.*, 2001; Kreiss *et al.*, 1999), relatively low immunogenicity (Whitmore, 1999; Dow *et al.*,

1999; Ruiz *et al.*, 2001; Whitmore, 2001). The application of *non-viral* plasmid-based gene medicine gives an attractive *in vivo* gene transfer strategy that is simple and lacks the kinds of risks that comes with a viral vector. Because most *non-viral* vectors are synthetic systems, the *non-viral* vectors are generally referred as the synthetic carriers. The advantages and disadvantages of viral and *non-viral* vectors are compared (see Table 1.3).

Table 1.3 The comparison between viral and *non-viral* vectors

	Advantages	Disadvantages
Viral vector	• High efficiency in gene delivery and expression. • Natural origin.	• Delivery of other diseases. • The uncertain long-term effect of the integrated transgene and of the virus in the host individual. • Low virus titer. • Restricted targeting of defined cell type specificity, limited size of incorporated DNA. • Manufacturing and packing problems. • Recombination. • High cost.
Non-viral vector	• They are not infectious. • Low degree of toxicity. • Easy control of their composition, simple analysis and production. • Able to large-scale production. • Flexibility in the size of the DNA to be delivered. • Relatively low immunogenicity.	• Low transfection efficiency. • Targeting is not specific. • Only transient expression. • Difficult *in vivo* application. • RES uptake

1.4.3. Synthetic vectors for gene delivery

Currently there is a rapid progress in the development of synthetic gene delivery systems because of their potential advantages, including no infectivity, low immunogenicity, flexibility in the size of the DNA to be delivered, well-defined characteristics, possibility of repeated clinical administration, and easy handling (Mahato *et al.*, 1999; Thomas and Klibanov, 2003; de Jong *et al.*, 2001; Kreiss *et al.*, 1999). Synthetic vectors are mainly formed by lipids, polymers or polypeptides, which usually form complexes with the therapeutic plasmid DNA.

One of the problems in gene delivery is that plasmid DNA is a large, negatively charged molecule up to 1 μm in size (Abdallah *et al.*, 1995). The cell membrane is negatively charged. Therefore, it is very difficult to internalize plasmid DNA into cells. In order to efficiently deliver plasmid DNA into cells it is necessary to condense the DNA to a much smaller size. Cationic vectors have the ability to ionically complex plasmid DNA into a more compact structure (Kabanov and Kabanov, 1995). This complexation enhances the *in vitro* cell uptake of plasmid DNA, resulting in a promoted gene transfection. The resulting complex not only protects the DNA from the attack of nucleases, but also changes the biological behavior of the complexed DNA. This may determine the biological fate of the DNA. Therefore, delivery systems can potentially improve transfection efficiency and specificity on different levels (condensation of DNA, cellular uptake, release from the endosome, as well as nuclear transport) through their interaction with the various biological barriers.

Lipid-based gene delivery

Liposomes are microscopic vesicles composed of uni- or multilamellar lipid bilayer surrounding aqueous compartments. Plasmids may be incorporated into liposomes to ensure protection against degradation by nucleases in biological fluids, to control disposition profiles, and to enhance intracellular delivery (Ellens *et al.*, 1984).

In 1987, Felgner and colleagues developed the artificial cationic lipid, Lipofectin™, that is composed of the cationic lipid (DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethyl- ammonium) and the colipid DOPE (dioleoylphosphatidylethanolamine). Since the introduction of the transfection reagent Lipofectin ™, many cationic lipid formulations have been used *in vitro* and *in vivo* transfection of plasmids. It is a noticeable improvement for gene delivery.

Cationic lipid reagents form small (average size 100-400 nm) unilamellar liposomes when formulated in water. Cationic lipids interact electrostatically with the negatively charged phosphate backbone of DNA and are attracted to the negatively charged surface of the cell membrane (Felgner *et al.*, 1987; Gareis *et al.*, 1991; Gershon *et al.*, 1993; Smith *et al.*, 1993).

The principle of delivery using a cationic lipid reagent is different from using neutral liposomes for transfections. With cationic lipid reagents, the plasmid is not encapsulated within the liposomes; rather, the negatively charged DNA binds spontaneously to the positively charged liposomes, forming cationic lipid-DNA complexes. Four liposomes associate with a single plasmid of about 5 kb (Felgner *et al.*, 1987). The cationic lipid-DNA complexes are delivered to cells. There are evidences that the mechanism of DNA delivery is through endosomes and lysosomes

(Coonrod *et al.*, 1997). Cationic lipid reagent-mediated transfection shows some special advantages, including high efficiency of DNA delivery in a wide range of cell lines, good reproducibility, and convenience.

As of November 2002, 12% of ongoing gene therapy clinical trials were based on lipid-DNA complexes (http://www.wiley.com/legacy/wileychi/genmed/clinical/) . The lipid-based gene delivery systems are probably the most popular method and have been used in human clinical trials. But lipid-based systems have some limitations, including lack of targeting, toxicity, low correlation between *in vitro* and *in vivo* transfection, poorly understood structure of DNA-lipid complexes, and variations arising during fabrication.

However, lack of targeting, toxicity and poor correlation between *in vitro* and *in vivo* transfection are the most critical drawbacks. The electrostatic interaction between the positively charged lipid-DNA complexes and the negatively charged cell membrane usually does not provide cell specificity. Cationic lipid mediated gene delivery systems lack target specificity that leads to low transfection efficiency in some tissues because of the interference with cationic lipid-binding macromolecules in the blood stream and in the extracellular matrix. Because the liposome is easily trapped by the RES (Alving *et al.*, 1978; Poste *et al.*, 1982; Fidler *et al.*, 1980), the targeting of liposomes to specific tissues has been difficult.

The toxicity of lipsosomes is another problem. The toxicity of DNA-lipid complexes is dose dependent (San *et al.*, 1993). At relatively low concentrations of DNA-lipid complexes, little or no toxic effects were reported after systemic or local injection of this complex in different animal models such as mice, rabbits, and pigs. At high

doses, acute inflammation occurred (Canonico *et al.*, 1994; Stewart *et al.*, 1992; Nabel *et al.*, 1992). The reason for the toxicity induced by cationic lipid is not very clear. Cationic lipids may not be readily metabolized or secreted. Consequently they may accumulate in the body and potentially produce unwanted side effects.

A low correlation between *in vitro* and *in vivo* transfection is a common disadvantage of *non-viral* gene delivery systems. *In vivo* positively charged complexes may interact with serum proteins, lipoproteins, heparin, and glycosaminoglycans in the extracellular matrix, leading to the aggregation or release of DNA from the complexes even before reaching the target cells. The poor correlation between *in vitro* and *in vivo* transfection activities of plasmid/lipid complexes may be in part due to different biological environments encompassing the cells *in vitro* and *in vivo* (Remy *et al.*, 1994).

Polymer-based gene delivery

The principle of polymer-based gene delivery systems is the same as for lipid-DNA complexes. The electrostatic attraction between the cationic charge on the polymer and the negatively charged DNA results in a particulate complex – the polyplex, which is the transfecting unit. Polycations interact with the polyanion DNA and condense it into compact particles (20–200 nm in diameter) (Dunlap *et al.*, 1997; Golan *et al.*, 1999; Liu *et al.*, 2001). The positively charged polyplexes also electrostatically interact with negatively charged proteoglycans of the cell membrane (Erbacher *et al.*, 1999), and then endocytosis may occur.

Compared with lipid-DNA complexes, polymer-based gene delivery is at an early stage, and it has not yet approached clinical trials. But after the introduction of polyethylenimine (PEI), a branched cationic polymer that has been shown to condense plasmid into colloidal particles, the development of polymer-based gene delivery system progresses very rapidly.

A lot of synthetic and natural polymers have been used as carriers for gene delivery. Synthetic polycations are peptides such as poly-l-lysine and poly-l-ornithine (Pouton *et al.*, 1998), as well as polyamines such as polypropylenimine, and polyamidoamine dendrimers (Qin *et al.*, 1998; Dennig and Duncan 2002; Zinselmeyer *et al.*, 2002). The natural polymers include proteins like histones (Balicki *et al.*, 2000; Esser *et al.*, 2000), gelatin (Truong-Le *et al.*, 1998) and cationized human serum albumin (Fischer *et al.*, 2001), as well as aminopolysaccharides such as chitosan (Borchard, 2001).

Lately, polymer-based controlled-release DNA delivery systems have been tested by some researchers. DNA was successfully encapsulated into biodegradable poly(D,L-lactide- *co*-glycolide) (PLGA) microparticles (Jones *et al.*, 1997) and PLGA microspheres (Ando *et al.*, 1999; Wang *et al.*, 1999; Luo *et al.*, 1999) for controlled DNA release. DNA has also been encapsulated into highly biocompatible poly(ethylene-*co*-vinyl acetate) (EVAc) matrices, resulting in the controlled and predictable release of bioactive DNA for several months (Luo *et al.*, 1999). This may lead to long-term release of DNA without the necessity for repeat administration.

Research has shown that polyplexes are potentially better than lipoplexes in many respects. Firstly, synthetic polymers often can be improved by chemical modification to achieve higher transfection efficiency or cell targeting without the loss of activity.

Polymers can be easily handled by chemical modification because they are made up by certain repeating structural units. For example, Choi and colleagues (Choi *et al.*, 1999) have conjugated linear polyethylene glycol (PEG) with a dense globular poly(L-lysine) (PLL) dendrimer forming PEG–block–PLL–dendrimer.

Secondly, the formation of the lipid-DNA complex involves both interaction among lipid molecules and interaction of lipid with DNA. The hydrophobic part of a lipid appears to be critical for the macroscopic characteristics of liposome formulations, such as their size, shape, and stability in the dispersed state, as well as interactions with other lipids, cell membranes, and DNA. This, in turn, affects the transfection efficiency (Smisterova *et al.*, 2001; Zuhorn *et al.*, 2002). Although these physical parameters are important, they are hard to control. The low ability to control these physical parameters induces the instability of their macroscopic properties (Simberg *et al.*, 2001) and thus limits their ability to be used as a gene vector. In contrast, the ability to control the macroscopic parameters of polyplexes is greater than lipoplexes because there is no interaction of the polycation molecules with each other.

However, polyplexes have the same limitation as lipoplexes: low correlation between *in vitro* and *in vivo* transfection. Compared to *in vitro*, *in vivo* gene delivery faces a lot of other obstacles like interactions with biological fluids in the extracellular matrix, and binding to a variety of non-target cell types. When the polyplexes are administered into the blood stream, they are placed in a complicated environment rich in negatively charged plasma proteins and circulating blood cells. As a result, the positive charge of the polyplexes is neutralized leading to changes in their biodistribution. But because easy handling of chemical manipulations makes the

incorporation of the cell-binding ligand possible, specific targeting of polymer-based gene vectors may also be achieved by this method.

All these considerations make polycations a compelling target for future exploration in *non-viral* gene delivery.

Peptide-based gene delivery

Peptides are the third group of materials, which have been applied as synthetic gene delivery systems.

Cationic peptides are able to bind/condense DNA and enhance cell uptake. The binding/condensation is, as in most other synthetic systems, through the electrostatic interaction between cationic amino acid residues and the anionic plasmid. For example, Schwartz and colleagues (Schwartz *et al.*, 1999) have synthesized short peptides derived from human histone or protamine and formed peptide–DNA–lipid complexes that enhanced DNA delivery both *in vitro* and *in vivo*.

Another approach was based on receptor-mediated uptake. DNA binding peptides can be synthesized that can be coupled to cell specific ligands, thereby allowing receptor mediated targeting of the peptide/DNA complexes to specific cell types. Positively charged macromolecules such as poly(L-lysine) (PLL), histones, protamine, or poly(L-ornithine) may be linked to a cell-specific ligand and then bound to plasmids via electrostatic interaction. The resulting complexes retain their ability to interact specifically with target cell receptors, leading to receptor-mediated internalization of the complex into the cells. Receptor ligands currently being investigated include glycoproteins (Wu and Wu, 1998; Findeis *et al.*, 1994), transferrin (Wagner *et al.*,

1990), insulin (Huckett *et al.*, 1990), epidermal growth factor (EGF) (Dai *et al.*, 2003), folate (Gottschalk *et al.*, 1994) and others. This method gives the potential to the site-specific delivery of plasmids. For example, PLL has been conjugated with ligands, such as asialoorosomucoid (ASOR), which binds to a liver-specific asialoglycoprotein receptor to achieve receptor-mediated uptake (Wu *et al.*, 1989). However, peptide-based gene delivery has its own limitations. For example, PLL is a representative of peptide-based gene delivery. The cationic peptide poly-L-lysine can condense DNA for more efficient uptake (Zauner *et al.*, 1998). However, the polydispersity of PLL preparations resulting from the procedures used in its synthesis leads to variable DNA delivery and difficulty in forming DNA complexes. In addition to its molecular heterogeneity, PLL is applied to living cells in nM concentrations, which limits its general applicability (Smith *et al.*, 1998). Furthermore, because site-specific gene delivery and expression are influenced by many factors such as the method of complexation, the extent of DNA condensation, the molecular weights of polycations and plasmid, and the number of ligand residues bound per polycation molecule (Erbacher *et al.*, 1995), and the ability of controlling site-specific delivery still need to be improved.

The above classification of vectors (lipid, polymer and protein-based gene delivery systems) is provided for convenience in comparisons; it is not meant that these methods must be independent. For example, polylysine, in combination with lipofectamine and DNA, exhibits higher transfection activity than the lipofectamine-

DNA complex alone (Vitiello *et al.*, 1996). Novel combinations of these methods may provide us with better DNA delivery systems in the future.

1.4.4. The application of nanoparticle in gene delivery

Currently, different kinds of nanoparticles are currently investigated as possible carriers for gene therapy (Coester *et al.*, 2000). Nanoparticles are defined as being submicronic (< 1 um) colloidal systems generally made of polymers (biodegradable or not). Based on the process used for the preparation of nanoparticles, nanospheres or nanocapsules can be synthesized (Lambert *et al.*, 2001).

Nanoparticles are one of the most promising vectors for gene therapy because of their special attractive features. Using nanoparticles the genetic information can be protected by encapsulation into the particle matrix, so bioavailability of the DNA can be improved. Additionally, ligands may be relatively easily conjugated to the nanosphere for targeting or stimulating receptor-mediated endocytosis; and the surface of the nanoparticles can be modified by coating or by chemical and/or immunological modification to improve the cell delivery and cell recognition (Coester *et al.*, 2000; Velge-Roussel *et al.*, 1996; Löbenberg *et al.*, 1998; Leong *et al.*, 1998).

Nanopaticles which are produced by various materials are applied in gene delivery as vector, including PLGA nanoparticles (Panyam *et al.*, 2002), biodegradable polyalkylcyanoacrylate nanoparticles (Fattal *et al.*, 1998; Chavany *et al.*, 1992), gelatin nanospheres, chitosan nanospheres (Leong *et al.*, 1998), sodium chloride-modified silica nanoparticles (Chen *et al.*, 2003), polystyrene nanoparticles (Sakthivel

and Florence, 2003), poly(l-lysine)/DNA nanoparticles (Parker *et al.*, 2002) and others.

1.4.5. Suitability of gelatin nanoparticles for gene delivery

Different kinds of materials are currently investigated as possible carriers for gene therapy. These materials are divided into two classes, synthetic and natural materials. Many synthetic materials are expensive and some of them are associated with cytotoxicity and are often toxic after repeated use; so prolonged *in vivo* usage is not feasible. Therefore we are focusing on natural polymers as DNA carriers.

From various natural polymers, we selected gelatin to make nanoparticles for gene delivery.

Firstly, as described in 1.2.2, gelatin is made form collagen. Collagen is the primary structural material of vertebrates and is the most abundant mammalian protein accounting for about 20–30% of total body proteins (Harkness, 1961). It takes part in the construction of organs and tissues and is involved in different functional expressions of cells.

Low toxicity is a major advantage of gelatin. The cytotoxicity of different cationic carriers was determined on COS-7 (green monkey kidney tumor cell) cell line, using the MTT dye reduction assay. Among the four vectors tested, i.e., gelatin, Lipofectamine, poly-L-lysine, and DEAE-dextran; gelatin was the least toxic (Brown *et al.*, 1995). Its biological safety has been also proven through the long history of applications in pharmaceutical and medical sciences (described in 1.2.6). The low

toxicity of gelatin may allow repeated administration of gelatin nanoparticles to compensate for a low transient transgene expression.

The low immunogenicity of gelatin has been discussed in 1.2.7.

Another advantage of gelatin is that gelatin can be easily chemically modified. The large number of pendant functional groups in the gelatin structure aid in chemical cross-linking and derivatization. For example, if needed, there exists the option to chemically introduce positive charges to the proteins. Positively charged, cationized gelatin can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. This feature may influence the efficiency of drug or DNA loading. Other functional groups like sulfhydryl group can also be introduced to the gelatin. The sulfhydryl groups are important because they are possible targets for the covalent linkage of drugs or antibodies to the particle surface. However, the surface of gelatin nanoparticles does not have a lot of sulfhydryl groups, because gelatin rarely contains less amino acids with sulfur-containing side chains. Weber *et al.* developed several ways to introduce sulfhydryl groups onto the surface of protein nanoparticles (Weber *et al.*, 2000b). Furthermore, ligands may be conjugated to gelatin nanoparticles allowing cell targeting.

In addition, the cost of producing this vector system is relatively low because gelatin is an inexpensive material.

Lastly, for industrial production, the system should be amenable to scale-up and manufacturing under the GMP guidelines.

These characteristics of gelatin nanoparticles make them a promising candidate as gene delivery vector.

Chapter 2

Hypothesis and Objectives

Based on the previous discussion, the following hypothesis was established for the current work:

- Gelatin nanoparticles are a potential DNA delivery system

To test this hypothesis, a number of specific experimental objectives were identified:

- To synthesize gelatin nanoparticles with defined size ranges and narrow size distribution
- To investigate the capabilities of gelatin nanoparticles to deliver model DNA (Lac Z gene) *in vitro*

Chapter 3

Materials and Methods

3.1. Preparation of nanoparticles

3.1.1. Materials

Gelatin type A from porcine skin (175 Bloom), gelatin type B from bovine skin (225 Bloom), and glutaraldehyde grade I 25% aqueous solution were obtained from Sigma Chemical Co (St Louis, MO, USA). Acetone and ethyl alcohol were purchased from Caleda (Georgetown, ON, Canada). All chemicals were of analytical grade and used as received.

3.1.2. Methods

The gelatin nanoparticles were prepared by a two-step desolvation method (Coester *et al.*, 2000). In brief, gelatin (1.25 g) was dissolved in 25 mL of distilled water. The solution was stirred at 600 rpm and heated at a constant temperature in a water bath until the gelatin was dissolved. Then 25 mL of acetone was added. The high molecular weight gelatin sedimented to the bottom while the low molecular weight gelatin remained in the supernatant. The supernatant containing the low molecular weight gelatin was discarded. The remaining sediment was redissolved in 25 mL of distilled water at a constant temperature. After the solution was adjusted to pH 2.5, about 75 mL acetone was added dropwise to the solution until nanoparticles were formed. Finally 400 μL of 25% glutaraldehyde solution was added as crosslinking agent (See Figure 3.1). After stirring for 12 hours, the remaining acetone was

evaporated using a rotary evaporator (IKA-Werk 7813 Staufen, Janke & Kunkel GmbHu. CoKG, Germany). The prepared nanoparticles were stored at 2-8°C for further experiments.

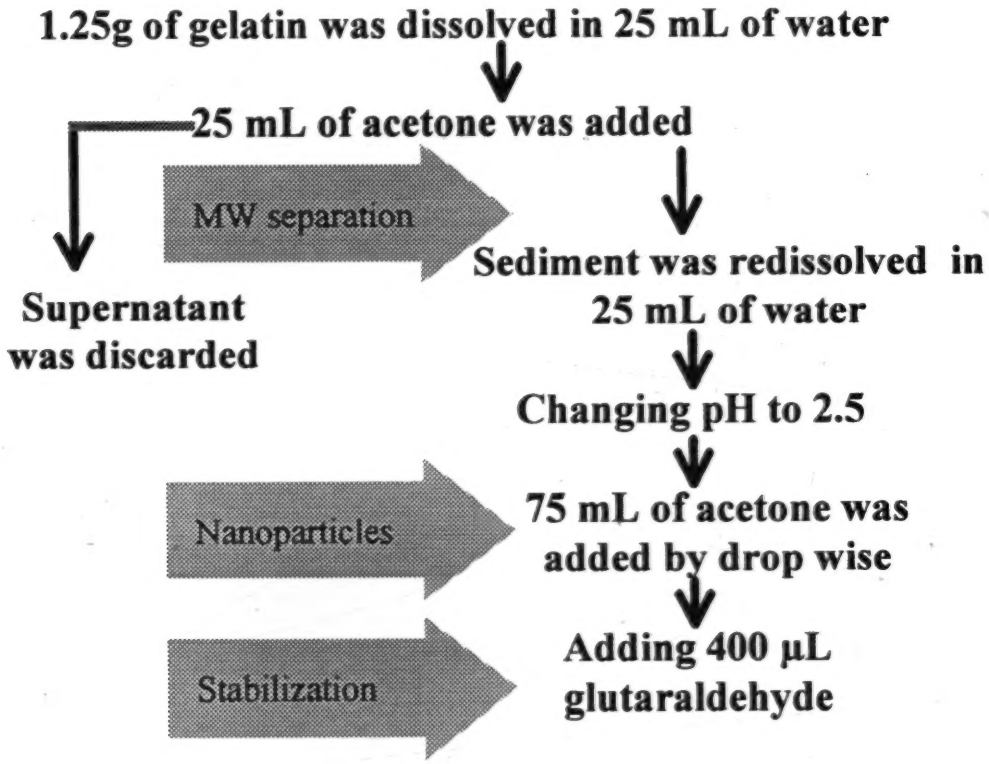
1.25g of gelatin was dissolved in 25 mL of water
25 mL of acetone was added
MW separation
Supernatant was discarded
Sediment was redissolved in 25 mL of water
Changing pH to 2.5
Nanoparticles
75 mL of acetone was added by drop wise
Stabilization
Adding 400 µL glutaraldehyde

Figure 3.1 The two-step desolvation method of preparing nanoparticles

3.2. Purification of nanoparticles

A 1.5 mL sample of nanoparticles was centrifuged at 16,000 x g for 10 minutes (Brinkmann Eppendorf centrifuge 5415). The particles were purified by three cycles of centrifugation and dispersion. The first two times they were washed using 1mL of

acetone/water (30/70) and the third time they were washed using 1mL of sterilized water. After the last centrifugation, the particles were redispersed in 0.5 mL of sterilized water or cell culture medium.

3.3. Gel permeation chromatography (GPC)

Gel permeation chromatography was performed using a HPLC system equipped with a HP 1100 series pump, Waters 410 differential refractomer, Precision Detectors PD-2000 DLS Dynamic Light Scattering Detector, and Precision Detectors software. The column used was a GPC-column YMC-Pack Diol-300 (300 x 8.0 mm, YMC Co., Ltd., Japan). PDI™ low dispersion in-line filter and PALL life Sciences Supor®-200 membrane filter (0.2 μm, 13 mm) were used in the system. The mobile phase was PBS (0.1 M) with a flow rate of 1 mL/min. The sample concentration was 5.0 mg/mL.

Precision Detectors PD2000/DLS Detector

The PD2000/DLS detector combines static and dynamic light-scattering (DLS) technologies to characterize both the molecular weight and size of molecules. The detector has a 10-μL flow-cell design and it can be coupled to size exclusion chromatography equipment in the lab. The schematic of static and DLS is seen in Figure 3.2.

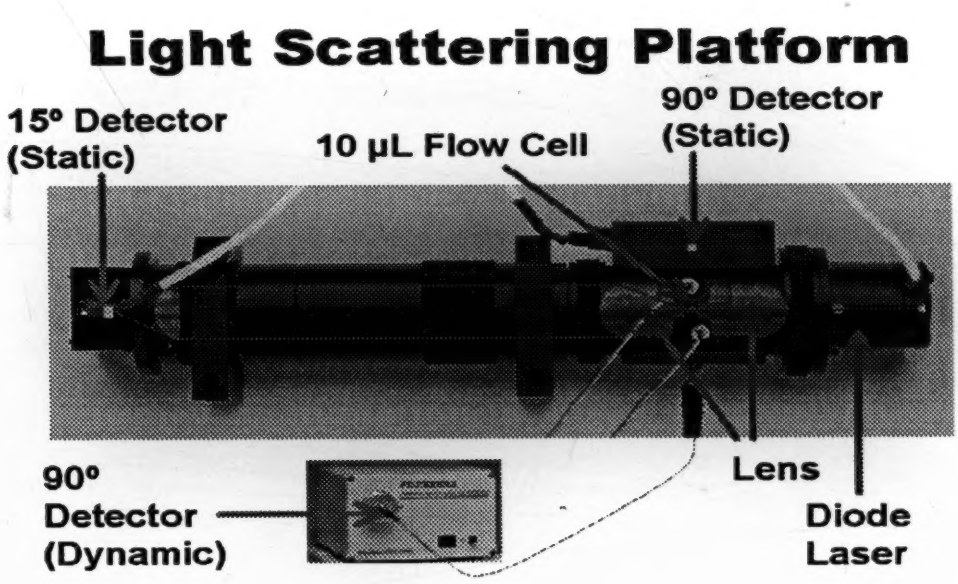

Figure 3.2 A combined static and dynamic light-scattering platform (modified from http://www.lightscatter.com)

Static light scattering is used for determining the molecular weight. The 90 degree and 15 degree laser light-scattering signals measure the absolute molecular weight of the eluting molecules. Dynamic light scattering determines the molecular size (hydrodynamic radius).

3.4. SDS-PAGE

3.4.1 Materials

1.5 M Tris-HCL, pH 6.8 or 8.8 (100 mL): 18.2 g of Tris was dissolved in 80 mL of water and the pH was adjusted to 6.8 or 8.8 using 1 M HCl. The solution was diluted to a final volume of 100 mL.

10% SDS (100 mL): 10 g of SDS was dissolved in 100 mL of deionized water.

7.5% Resolving gel (20 mL): 3.76 mL of 40% Acrylamide: bisacrylamide, 5 mL of 1.5 M Tris-HCl (pH 8.8), 200 µL of 10% SDS, 10.94 mL of water, 100 µL of 10% ammonium persulfate and 10 µL of TEMED were mixed to get the resolving gel.

4% Stacking gel (5 mL): 0.5 mL of 40% Acrylamide: bisacrylamide, 1.26 mL of 1.5 M Tris-HCl (pH 6.8), 50 µL of 10% SDS, 3.18 mL of water, 25 µL of 10% ammonium persulfate and 5 µL of TEMED were mixed to get the stacking gel.

4 x SDS gel-loading buffer (10 mL): 2.5 mL of 1 M Tris buffer, 4 mL of glycerol, 0.8 g of SDS, 0.4 mL of 2-ME, and 0.1 mg of bromphenol blue were mixed together. The volume was adjusted to 10 mL with H_2O. The solution was stored at –20°C in 1 mL aliquots.

Tris-glycine electrophoresis buffer (pH 8.3): 15 g of Tris, 72 g of glycine and 5 g of SDS were dissolved in 1 L of water and the pH was adjusted to 8.3 using 1 M of HCL. It was diluted five times at the time of use.

Staining solution: Dissolve 0.25 g of Commassie Brilliant Blue R250 in 90 mL of methanol:H_2O (1:1 v/v) and 10 mL of glacial acetic acid. The solution was filtered and stored at room temperature.

De-staining solution: 25% methanol and 7% acetic acid were mixed in water.

Sample preparation: 10 μL of gelatin (20 μg/μL), 10 μL of 4x SDS gel-loading buffer, and 20 μL of water were mixed together in micro tubes and heated them to 100°C for 10 minutes to denature the proteins.

3.4.2 Methods

SDS-PAGE was used to determine the molecular weight distribution of gelatin. Firstly, the glass plates were assembled. The separating gel solution was poured into the gap between the glass plates (enough space should be left for the stacking gel). Butanol was added to overlay the top of the separating gel. The gel was placed in a vertical position at room temperature. After the polymerization was completed (30 minutes), the overlay was poured off and deionized water was used to wash the top of the gel several times to remove any unpolymerized acrylamide. The stacking gel solution was directly poured onto the surface of the polymerized resolving gel. A Telfon comb was immediately inserted into the stacking gel solution followed by the slow addition of the stacking gel solution to fill the rest of the space. The gel was placed in a vertical position at room temperature.

After the polymerization was completed (30 minutes), the Teflon comb was carefully removed and the wells were washed with deionized water to remove any unpolymerized acrylamide. Then the gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the reservoir. The samples were loaded in the wells. The electrophoresis apparatus was attached to an electric

power supply. A voltage of 8V/cm was applied to the gel. After the dye front moved to the resolving gel, the voltage was increased to 15V/cm and the samples were run until the bromophenol blue reached the bottom of the resolving gel. The power supply was disconnected and the gel was washed with water. The gel was dipped into staining solution and placed on a shaker rotating 1 hour for staining, followed by immersing it in a destaining solution overnight to remove the background. The gel was wrapped and dried using a porous plastic.

3.5. Characterization of nanoparticles

3.5.1. Measurement of nanoparticle size and size distribution

A Zetasizer (HSA3000, Malvern, England) was used to measure the particle size distribution and the polydispersity index. In this instrument, the polydispesity index term equals the intensity-weighted variance divided by the intensity weighed average of the diffusion coefficient distribution squared. Measurements were carried out at 25°C. For particle size analysis, 2.5 mL of fresh filtered (0.45 μm) water was filled into a single-use cuvette. A portion of the sample (normally 10-100 μL) was added to the cuvette until the solution appeared slightly turbid. To determine the size of the nanoparticles, the Zetasizer uses a laser beam, which is scattered by the nanopariticles in suspension. It then measures the light scattered at a 90 degree angle. The Zetasizer accumulates data from 10 runs and the average of the data is used to calculate the nanoparticle size.

3.5.2. Scanning-electron-microscopy (SEM) of gelatin nanoparticles

The nanoparticle suspension was air-dried and mounted on an aluminum sample mount and sputter coated with chromium to minimize surface charging. The samples were viewed by scanning-electron-microscopy (SEM) (JEOL 6301F, Field Emission Scanning Electron Microscope) to provide high-resolution digital images.

3.5.3. pH dependent zeta potential of nanoparticles

A Zetasizer model HSA 3000 (Malvern, England) equipped with a capillary cell and an autotitration unit determined the surface charge of the nanoparticles. About 1 mL of nanoparticle suspension was diluted with 1 mM NaCl solution. The NaCl solution compensated for the conductivity effect resulting from the addition of HCl or NaOH in pH-titration. The initial sample volume was 50 mL. The concentration of the sample can be checked by the count rate: The normal range of count rate is 10-3000KCps. The optimum range of 50-300 KCps was chosen in our measurements. In the measurement, the pH value was adjusted from 2.0 to 11.0 with 0.25 M HCl and NaOH using an autotitrator. The electric field was applied to observe the electrophoretic velocity of the particles. All the measurements were done at room temperature.

3.6. DNA binding

3.6.1. Materials

50 x Tris-acetate (TAE) Buffer: 242 g Tris was dissolved in 500 mL H_2O. 100 mL 0.5 M of Na_2EDTA (pH 8.0) and 57.1 mL of glacial acetic acid were added. Then the volume was adjusted to 1 L with water. The solution was stored at room temperature.

Preparing samples: Incubating different amounts of nanoparticles with DNA (300 bp) for 30 minutes at 37°C (see Table 3.1 and 3.2).

Table 3.1 Gelatin A nanoparticles with DNA

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Gelatin A nanoparticles (µL)	2.5 (2.8 µg/µL)	5 (2.8 µg/µL)	10 (2.8 µg/µL)	6 (10.1 µg/µL)	9 (10. µg/µL)
DNA (µL) 3 ng/µL	10	10	10	10	10
H_2O (µL)	7.5	5	-	4	1
DNA: Nanoparticles	1:250	1:500	1:1000	1:2000	1:3000

Table 3.2 Gelatin B nanoparticles with DNA

	Sample 1	Sample 2	Sample 3
Gelatin B nanoparticles (µL) (2.8 µg/µL)	2.5	5	10
DNA (µL) 3 ng/µL	10	10	10
H_2O (µL)	7.5	5	-
DNA: Nanopartilce	1:250	1:500	1:1000

3.6.2. Methods

The DNA binding capacity was conformed by agarose gel electrophoresis.

To prepare 100 mL of a 1.2% agarose solution, 1.2 g agarose was put into a glass beaker or flask following by addition of 100 mL 1X TAE. The slurry was stirred on a hot plate until the agarose was dissolved and solution was clear. The solution was cooled to about 55°C. The gel tray was prepared by sealing the ends with tape. Gel solution (50 mL) was poured into tray to a depth of about 5 mm. A comb was placed in the gel tray about 2.5 cm from one end of the tray and the comb was positioned vertically such that the teeth are about 1-2 mm above the surface of the tray. The gel was solidified for about 20 minutes at room temperature. Then the comb and tape were gently removed. The tray was placed in electrophoresis chamber and covered (just until wells were submerged) with electrophoresis buffer (the same buffer used to prepare the agarose 1X TAE). After that, 5 µL of 5x loading buffer was added for every sample. Loading buffer was purchased from Sigma-Aldrich. 10 µL of 3 ng/µL DNA was mixed with 2.5 µL of 5x loading buffer as reference. The samples, the reference and the DNA marker were loaded to wells. Around 120 volts were applied until the dye markers had migrated an appropriate distance. Finally, the gel was stained in 0.5 µg/mL ethidium bromide until the DNA had taken up the dye and was visible under UV light, if the DNA was not to be used further.

3.7. Cell culture

COS-1 (African green monkey tumor cell), 143B (human osteosarcoma cell), HELA (human adenocarcinoma cell) and 293 (human kidney tumor cell) cell lines were used for the gene transfection studies.

COS-1 was propagated in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose. HELA and 293 cells were grown in Eagle Minimum essential medium (EMEM) 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. 143B were maintained in MEM, Minimum essential medium (Eagle) in Earle's BSS with 0.015 mg/mL 5-bromo-2'-deoxyuridine. The media were purchased from the GIBCO BRL Co. All media contained 1% (v/v) Antibiotic-Antimycotic (GIBCO BRL). The media of COS-1, 143B and HELA cell lines contained 10% (v/v) fetal bovine serum (FBS). The medium of 293 cell line contained 10% (v/v) heat-inactivated horse serum. Fetal bovine serum and horse serum were purchased from Sigma (St Louis, MO, USA). The cells were grown in an atmosphere of 5% CO_2 and 100% relative humidity and passaged every 4-5 days.

3.8. MTT cytotoxicity assay

Studies were carried out with COS-1 or 143B cells that were routinely cultured in complete DMEM or EMEM medium supplemented with 10% fetal bovine serum (FBS). Exponentially growing cells were trypsinized, centrifuged and suspended in

growth medium and readjusted to $16x10^3$ cells /mL. The cells were seeded into 96 – well plates at $16x10^2$ cells /well and incubated in 37°C, 5% CO_2 for 24 hours.

The naked gelatin A nanoparticle suspension in DMEM or EMEM medium was added to the wells at a volume of 100 µL to produce a final concentration of design. Control wells were filled with 100 µL of DMEM medium. The plates were incubated for 3-5 days at 37°C in humidified atmosphere containing 95% air and 5% CO_2. At the end of incubation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) was dissolved in PBS at 5 mg/mL, filtered through a 0.45 µm membrane filter and diluted 1:5 in pre-warmed DMEM or EMEM medium then added 50 µL to each well. The plates were incubated at 37°C for 4 hours. The medium was then removed from wells and 150 µL of dimethyl sulfoxide was added to each well. The plates were on a shaker for 15 minutes to dissolve the formazan crystals and then read immediately at 540 nm on a scanning multi-well spectrophotometer (EL312e Bio-Kinetics Microplate Reader, Bio-Tek instruments).

3.9. Transfection studies

3.9.1. Materials

Plasmid. The DNA-pcDNA3.1/LacZ obtained from Invitrogen was used to express β-galactosidase. The DNA was stored at –20°C.

HEPES buffer solution (1M) was purchased from Sigma-Aldrich, which contains HEPES (*N*-2-hydroxyethylpiperazine-*N* '-2-ethane sulfonic acid 238 g/L) prepared with distilled water. The solution had pH 7.2 to 7.5 and pKa 7.3 at 37°C. It was stored

in 2°C to 8°C. The solution HEPES-DMEM used in transfection was DMEM liquid medium containing 20 mM HEPES buffer solution. The solution HEPES-EMEM used in transfection was EMEM liquid medium containing 20 mM HEPES buffer solution.

PBS (Phosphate Buffered Saline): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 were dissolved in water. Then the solution was diluted to the final volume of 1 L with distilled water. The final pH was kept at 7.4 and the solution was autoclaved and stored at room temperature.

X-gal buffer (100 mL): 1 mL of 1 M Na_2HPO_4, 0.1 mL of 1 M $MgCl_2$, 15 mL of 1 M NaCl, 3.3 mL of 100 mM $K_4Fe(CN)_6.3H_2O$, 3.3 mL of 100 mM $K_3Fe(CN)_6$, and 77.24 mL of H_2O were mixed together. The concentrations of different compositions were shown in Table 3.3.

Table 3.3 X-gal buffer compositions

Composition	Concentration (mM)
Na_2HPO_4	10
$MgCl_2$	1
NaCl	150
$K_4Fe(CN)_6.3H_2O$	3.3
$K_3Fe(CN)_6$	3.3

3.9.2. Method

Preparation of DNA-nanoparticle complexes and DNA-control complexes:

The following experiments were performed in 24-well plates. Each well received 100 μL of transfection solution, which contains 1 μg of DNA.

DNA-nanoparticle complexes were prepared in micro tubes by mixing 1 μg of DNA (20 μL, 0.05 μg/μL) with gelatin A nanoparticles (46 μL, 20.2 μg/μL) together, then diluting it to a final volume of 150 μL by HEPES-DMEM (COS-1 cell line) or HEPES-EMEM (143B, Hela and 293 cell lines).

Positive control were prepared in micro tubes by mixing 1 μg of DNA (20 μL, 0.05 μg/μL) with 1 μL of Lipofectamine reagent (2mg/mL) which was purchased from Gibco BRL, then adding 129 μL of HEPES-DMEM or HEPES-EMEM to a final volume of 150 μL.

Negative control only consisted of 1 μg of DNA (20 μL, 0.05 μg/μL). Then it was diluted to a final volume of 150 μL by HEPES-DMEM or HEPES-EMEM.

All samples were kept for 30 min at room temperature, and then used as transfection solutions.

The day before transfection, the cells were trypsinized. First, the medium was removed from the flask, and about 5 mL PBS solution was added to cover the cell monolayer. The flask was swirled to distribute the solution evenly, and then the buffer was removed and discarded. The washing procedure was repeated once. 1- 2 mL of 1 x trypsin-EDTA solution (10 x trypsin-EDTA purchased from Gibco BRL) was added to cover the monolayer and the flask was rocked 4-5 times to coat the monolayer. The

flask was placed in a CO_2 incubator at 37°C for 1-2 min until the cells just began to detach. The cells were viewed under a microscope to check whether cells were detached from the grow surface. When cells were detached, medium-containing serum (C-DMEM, 8 mL) was added to the cells to inactivate the trypsin. The cells were gently pipted up and down to break cell clumps. Then the mixture was centrifuged at 1,000-x g for 10 min and the supernatant was discarded. The pellet was re-suspended in 8 mL of C-DMEM. After that, the cells were counted using hemacytometer, and plated in a 24-well plate so that they are 70-80% confluent on the day of transfection. The cells were incubated in an incubator at 37°C and 5% CO_2.

On the day of tansfection, gently aspirate the growth medium from the wells, and wash cells once with PBS. After that, the DNA-nanoparicle mixtures 150 uL and 100 uL of growth medium without serum and antibiotics are added to the cells. The plate was gently swirled to ensure uniform distribution of the complexes. The cells were incubated with the complex at 37°C for 3 hours. After 3 hours, the DNA-nanoparicle mixture and the growth medium without serum and antibiotics were replaced with completed medium containing serum and antibiotics (1 mL/well). The cells were further incubated at 37°C for 24 hours.

For *in situ* β-gal staining of transfected cells, the cell are washed twice with PBS and then fixed with 0.25% glutaraldehyde (0.5 mL/well) for 15 minutes at room temperature. After that, the glutaraldehyde solution was gently aspirated from the wells, and the cells were washed with PBS. A stock solution of 20 mg/mL X-gal in dimethylformamide was stored at –20°C in polypropylene tube in the dark. 0.25 mL of 0.2 mg/mL X-gal in X-gal buffer was freshly prepared and added into each well.

The cells were incubated for 2 hours at 37°C. To evaluate the proportion of blue cells (transfected cells), the cells were observed on microscope. For the storage of plates, the glutaraldehyde solution was aspirated from the wells, and PBS was used to rinse cells. Each well was fixed with 1 mL of 10% buffered formalin phosphate for 10 minutes at room temperature. Rinse with PBS and store in PBS at 4°C.

3.10. Statistical analysis

The effect of different amounts of glutaraldehyde on the synthesis of nanoparticles was compared by ANOVA and Tukey.

Chapter 4

Results and discussion

4.1. Preparation of nanoparticles

One of the objectives in this project was to synthesize the nanoparticles with defined size range and narrow size distribution. A two-step desolvation method that was published by Coester *et al.* in 2000 was used to synthesize nanoparticles. In order to optimizing this method, different parameters such as molecular weight, pH at the second desolvation step, type of desolvating agent, amount of glutaraldehyde, temperature, and nature of gelatin were investigated and the effect of these parameters on the particle size and the polydispersity index of nanoparticles were studied.

Molecular weight

The molecular weight of gelatin is an important parameter of making nanoparticles. If the gelatin nanoparticles were prepared by a one-step desolvation procedure, this method often leads to the formation of large aggregates. The formation of large aggregates can be decreased by using a two-step desolvation procedure. In the first desolvation step, the high molecular weight gelatin was roughly separated from the low molecular weight molecules. The gel permeation chromatography result showed that the sediment on the bottom was made up by mainly high molecular weight gelatin, while the supernatant was mostly make up by the low molecular weight gelatin (Coester *et al.*, 2000). The supernatant was discarded. The removal of the low molecular weight gelatin fraction in the supernatant after the first desolvation step

reduced the formation of aggregates during crosslinking and prevented further secondary aggregation and flocculation of particles during storage (Coester *et al.*, 2000). The gelatin nanoparticles prepared by the two-step desolvation methods were stable. In Coester's paper, he described that the nanoparticles were stable for three months. In our lab, the nanoparticles were stored for about one year and the particle size was constant.

Because of the important effect of molecular weight, two methods, gel permeation chromatography and SDS-page, were used to investigate the molecular weight distribution of gelatin.

(1) Gel permeation chromatography

Commercial gelatins are a protein mixture of polypeptide chains with different molecular weights. The range of gelatin molecular weight is very large and ranges from a few thousand up to several hundred thousand Daltons (Veis, 1964). Based on this information, a column with wide separating range was selected. And because of the high sensitivity of the laser light scattering detector, a low bleeding column was used to reduce the noise. The manufacture of the detector suggested to use the columns made by two companies-YMC or Polymer Laboratories. Based on our requirement, the YMC-Pack Diol-300 was chosen. The separating range of this column is for a molecular weight range of 10,000 to 1,000,000. This separating range seemed to suit our separation requirement for gelatin. However, because of the huge molecular weight distribution of gelatin, this column did not offer enough separation. It cannot sufficiently separate the molecular weight of the different gelatin fractions,

although the composition of mobile phase, pH of mobile phase, flow rate and temperature were changed. As result we were only able to confirm the large molecular weight range of gelatin using the laser light scattering detection method. The polydispersity value (polydispersity index is the ratio of the weight-average molecular weight to the number-average molecular weight) of gelatin B was determined to be 81.330. Compared with many other samples, this value is too big. For example, many biopolymers are monodisperse and their polydispersity index would have a value of one. For vinyl polymers, the polydispersity index can be about 10 (Martin *et al.*, 1983). Compared with these results, the polydispersity value of gelatin that we got is very big and this value showed that gelatin had a huge molecular weight distribution.

(2) SDS-page

Another method to investigate the molecular size distribution of biomaterials is gel electrophoresis. The result from the SDS-page (see Figure 4.1) demonstrated that the gelatin fraction in the sediment was still a mixture of high and low molecular weight gelatin, although a lot of low molecular weight gelatin was already discarded by the first desolvation step. That means in the two-step desolvation method, the molecular weight separation is not exclusive.

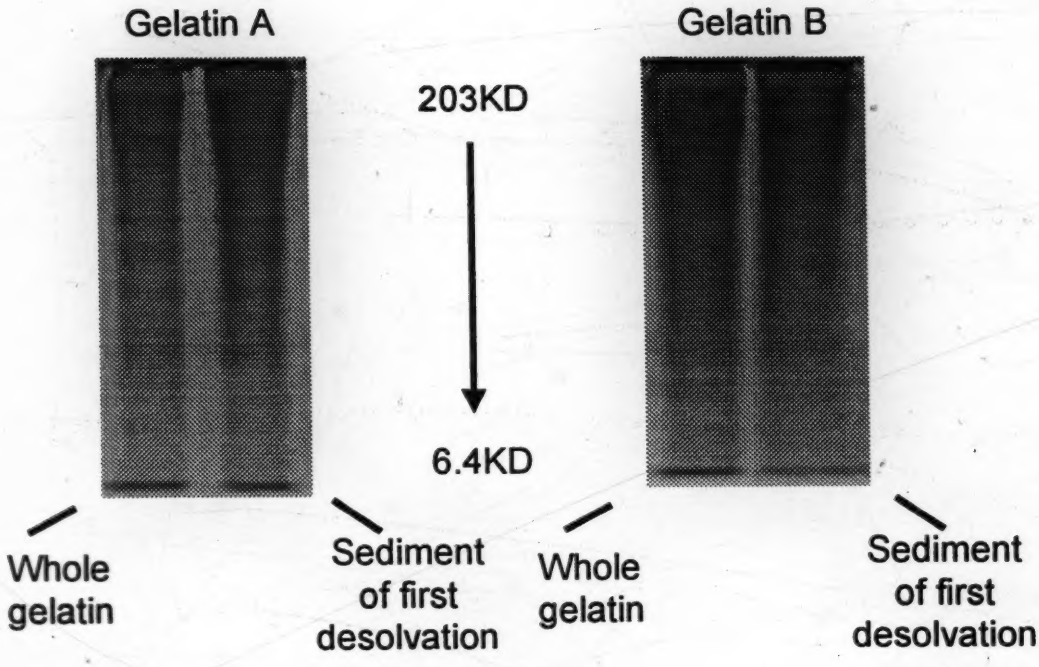

Figure 4.1 Molecular weight of gelatin (SDS-PAGE)

In the beginning of the project, we assumed that we would able to separate the high molecular weight gelatin clearly from low molecular weight gelatin. This might improve the synthesis of small nanoparticles. The hypothesis was that highly separated gelatin fractions improve the synthesis of nanoparticles (smaller size and low polydispersity index). Therefore, a three-step desolvation method was designed to improve the molecular weight separation (See Figure 4.2). But the result showed that the mean size of nanoparticles by the three-step desolvation method was increased by about 120 nm to 281.3 nm compared to the two-step desolvation method) and the polydispersity index was increased to 0.388 compared to 0.0332

using the two-step desolvation method). Based on this result, the three-step desolvation method did not improve the method of preparing nanoparticles. We concluded from this experiment that a small fraction of low molecular weight gelatin might be essential to form nanoparticles.

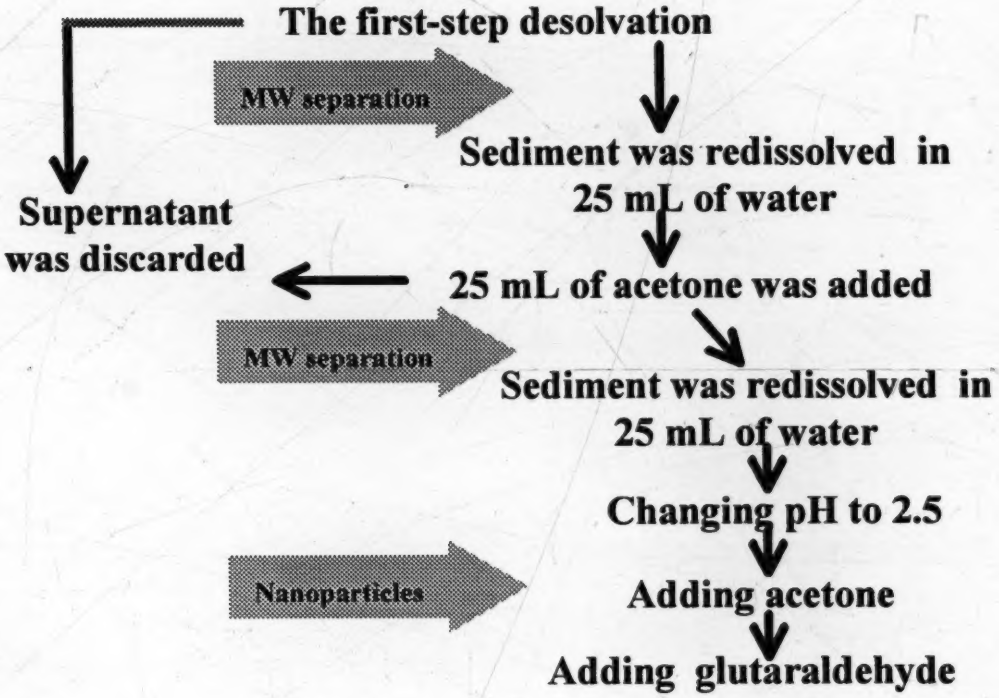

Figure 4.2 The three-step desolvation method of preparing nanoparticles

pH

The data in Table 4.1 show the result of the particle size changes at different pH values.

Table 4.1 The effect of pH on the synthesis of nanoparticles (n = 5)

pH	2.5	4-11	12
Size (nm)	120-140	Aggregates	360-380

In the pH range of 4-11, when a desolvating agent was added, yellow or white aggregates were formed. These aggregates were observed by visual inspection. At pH 12, nanoparticles were successfully produced, but their size was increased. pH 2.5 was the best pH for preparing gelatin A and B nanoparticles.

Desolvating agent

Ethanol and acetone were used as the desolvating agents. Nanoparticles under 100 nm were prepared using ethanol. Nanoparticles above 100 nm were prepared using acetone.

Amount of glutaraldehyde

To investigate the effect of the amount of the crosslinker, 100, 200, 300, 400 and 500 µl of a 25% aqueous glutaraldehyde solution were added to the nanoparticles in the final step of the synthesis process. The results are shown in Table 4.2.

Table 4.2 The effect of the amount of glutaraldehyde on the synthesis of nanoparticles (n = 3)

Glutaraldehyde amount (µL)	Size (nm)	Polydispersity Index
100	384±15	0.339±0.088
200	213±15	0.0453±0.023
300	199±22	0.0722±0.022
400	197+17	0.0357±0.012
500	202±21	0.0681±0.021

The comparison of the data indicates, with 95% confidence that there are significant differences on the size and polydispersity index of nanoparticles among different amount of glutaraldehyde (ANOVA). However, the data for using 200 – 500 μL of glutaraldehyde are not significantly different. They are significantly different compared to nanoparticles crosslinked by 100 μL of glutaraldehyde (Tukey).

Nature of gelatin and temperature

The nature of gelatin had also the influence on the particle size. At 40 and 50°C the size of gelatin A nanoparticles was bigger compared to gelatin B nanoparticles. The effect of temperature on the particle size was studied too. The results of 40, 50 and 60°C showed that the size of nanoparticles increased when the temperature was increased (see Table 4.3).

Table 4.3 The effect of the nature of gelatin and temperature on the synthesis of nanoparticles (n = 5)

Type of gelatin	Temperature (°C)	Size (nm)
B	40	40-140
A	40	200-230
B	50	180-240
A	50	200-300
B or A	60	220-350

Discussion

Our systematic investigation of the synthesis parameters showed that controlling the experimental conditions, nanoparticles with defined size ranges and narrow size distribution can be synthesized. Molecular weight, pH at the second desolvation step,

type of desolvating agent, amount of glutaraldehyde, temperature, and nature of gelatin were the critical parameters for the synthesis of gelatin nanoparticles. The smallest particles were prepared using gelatin B at 40 °C using ethanol as the desolvating agent.

The molecular weight of gelatin is an important parameter for preparing nanoparticles and has effect on the size of nanoparticles. Similar result was shown in preparation of collagen microparticles (Rossler *et al.*, 1995), the particle size was mainly controlled by the molecular weight of the collagen used in the preparation.

To produce nanoparticles, the pH of the gelatin solution in the second desolvation step should be far away from the isoelectric point. When the pH is far away from the isoelectric point, the particles have a higher zeta potential. When the pH is close to the isoelectric point, the particles have lower zeta potential. The zeta potential is the difference in potential between the surface of the tightly bound layer (shear plane) and the electro neutral region of the solution. The zeta potential has an effect on the stability of systems because this potential decides the degree of repulsion between similar charged dispersed particles. If the zeta potential is reduced below a certain value, the attractive forces exceed the repulsive forces, and the particles are formed (Martin *et al.*, 1983). In our system, aggregates formed at pH 4-11. This result might be due to the charge of the gelatin molecules at a pH close to the isoelectric point. The formation of particles seemed in this pH range difficult to be controlled when the desolvating agent was added. However, if the pH was far away the isoelectric point, the slow addition of desolvating agent was able to control particle synthesis.

Different size distributions of the resulting nanoparticles were obtained using acetone and ethanol. This result might be due to the different polarity of these two desolvating agents. Compared with acetone, ethanol is more polar and the desolvation process might be slower and therefore better controlled using ethanol as desolvating agent.

There was a significant difference in nanoparticle size distribution if only 100 µL of glutaraldehyde were used as crosslinking agent compared to 200-500 µL of glutaraldehyde. In the latter case no statistical difference was observed between the different nanoparticle batches. However, if only 100 µL were used the size increased. We think that this observation is due to an inefficient crosslinking of the particles. The gelatin molecules were able to swell in aqueous more compared to the nanoparticles prepared with higher concentrations of crosslinker.

The size difference between gelatin A and B nanoparticles may also due to the different molecular weight distributions of gelatin A and B as shown by the SDS-page in Figure 4.1.

In our result, the size of the nanoparticles increased when the temperature was increased. When the temperature increased, the movement of the gelatin molecules in the solvent increased too. This seems to influence the formation of nanoparticles at higher temperatures. As a result, nanoparticles with the bigger size were formed.

4.2. Characterization of nanoparticles

Particle size and size distribution of the nanoparticles

The size and size distribution of the nanoparticles was determined using a Zetasizer. The particle size distribution was interpreted in three different ways, intensity,

volume and number (see Table 4.4). The intensity model was used in our measurement because the intensity model is the most accurate one. The volume and number models are converted from the intensity model. Figure 4.3 shows the smallest nanoparticles that were synthesized in our lab until now. In this figure, % in class represented relative intensity. This sample was produced using gelatin B at 40°C by ethanol as the desolvating agent. The mean diameter of nanoparticles was 43.7 nm. The polydispersity index is 0.209.

Table 4.4 Output of the measured the particle size distribution using a Zatasizer

Size(nm)	Intensity	Volume	Number
15.5	0	0	0
19.5	0	1.7	4.4
24.5	1.8	13.2	21.9
30.9	21.3	30.8	36.7
38.9	40.7	32.5	26.8
49	32	17.2	8.8
61.7	4.2	4.3	1.3
77.7	0	0.2	0
97.8	0	0	0

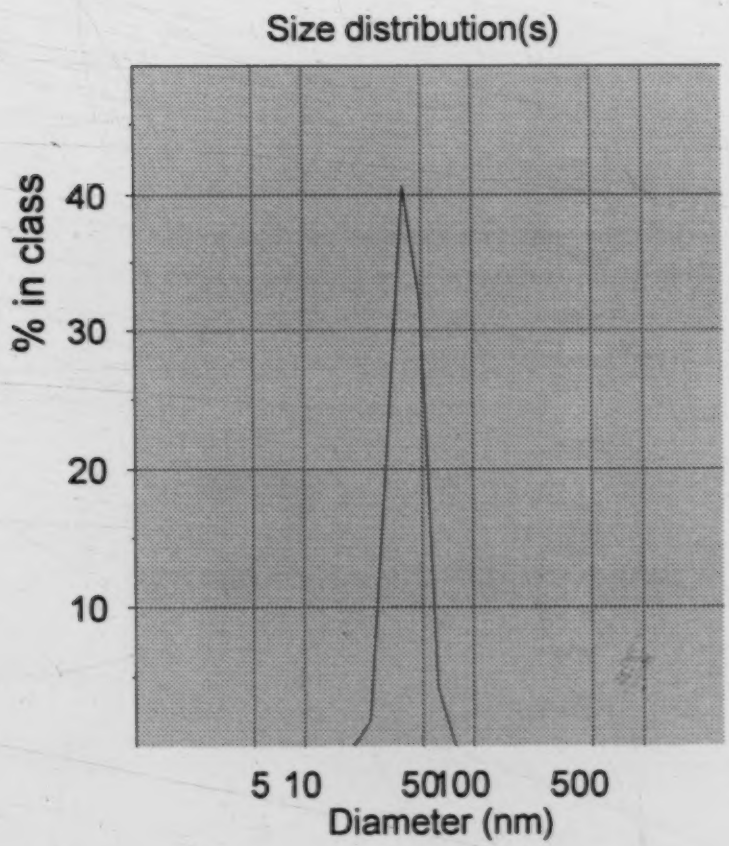

Figure 4.3 The smallest gelatin nanoparticles that were synthesized in our lab measured by laser light scattering

Scanning-electron-microscopy (SEM) of gelatin nanoparticles

The Figure 4.4 shows the scanning-electron-microscopy (SEM) picture of gelatin B nanoparticles in Figure 4.3. The SEM picture showed that the particle size was 43nm ±2.1nm. This result confirmed the size that was measured by Zetasizer.

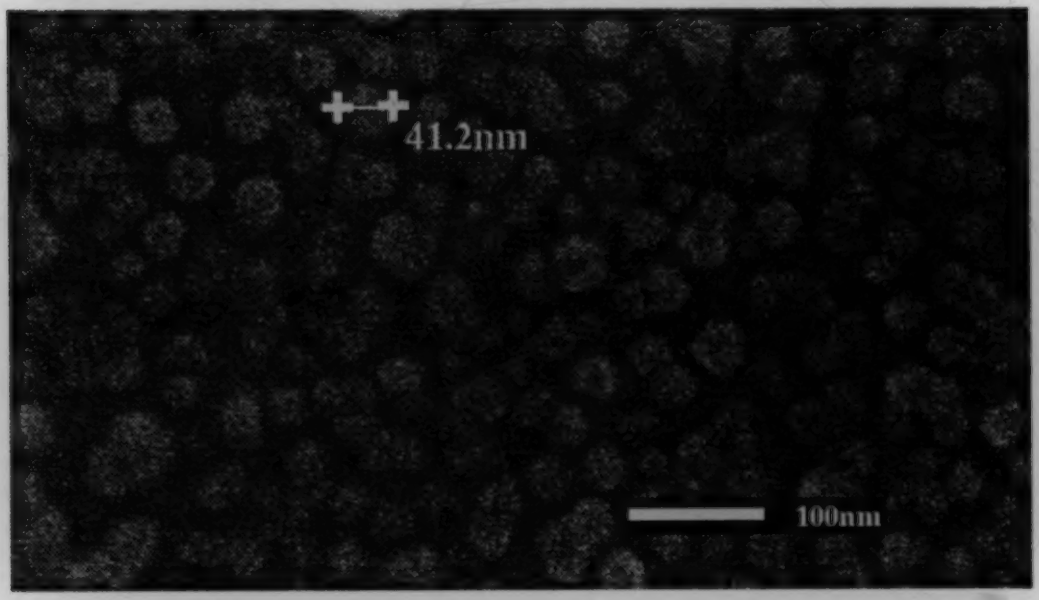

Figure 4.4 The scanning-electron-microscopy (SEM) picture of gelatin nanoparticles

pH-titration of gelatin A and B nanoparticles

The zeta potential of the nanoparticles was determined using a Zetasizer. The zeta potential of gelatin A and B nanoparticles were compared in a pH range of 2 to 11. The results in Figure 4.5 show the pH-titration of the nanoparticles at 9 different pH values. Increasing the pH of the nanoparticle suspension decreases the zeta potential. As the pH increases gelatin nanoparticles becomes more negative because carboxyl

groups get de-protonated. Conversely, gelatin nanoparticles are more positively charged at the lower pH leveles because of the protonation of amino groups. The zeta potential of gelatin nanoparticles seems to be a function of the pH of the medium.

The isoelectric point of gelatin A and B nanoparticles was 6.23 and 5.03 respectively. At the isoelectric point, the zeta potential values are zero. In comparison, the zeta potential of gelatin A nanoparticles is generally higher than gelatin B nanoparticles. The differences in the zeta potential between the two kinds of nanoparticles is due to the chemical extraction of gelatin A and B, and corresponds to the pI of the gelatin.

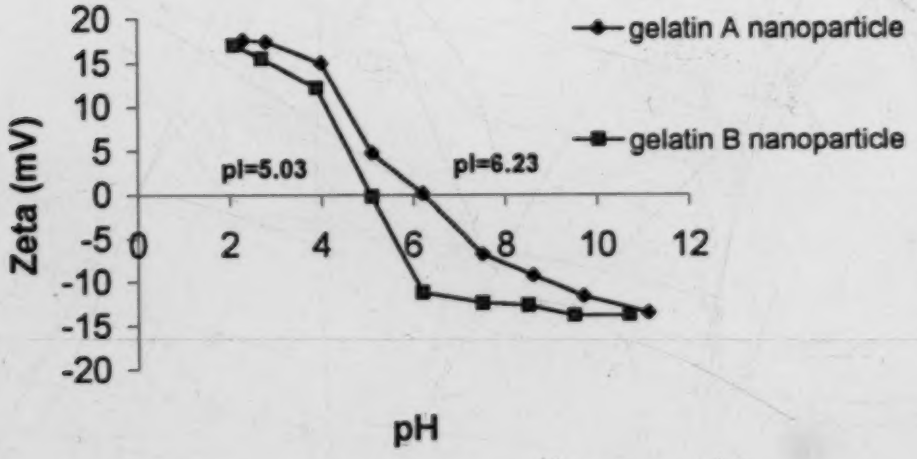

Figure 4.5 pH-titration of gelatin A and B nanoparticles

4.3. DNA binding study

4.3.1. Results

The result from DNA binding study showed that the DNA loading capacities of gelatin A and gelatin B nanoparticles were different. The pictures of agarose gel

electrophoresis (see Figure 4.6) demonstrated that gelatin A nanoparticles adsorbed DNA to their surface. A serial of ratios (w/w) of DNA and gelatin A nanoparticles (1:250, 1:500, 1:1000, 1:2000 and 1:3000) was tested. In the 1:1000, 1:2000 and 1:3000, free DNA was not detected. This indicated that DNA almost completely attached to the surface of gelatin A nanoparticles from this ratio. For gelatin B nanoparticles, there was free DNA that could be detected in the different ratios of DNA and nanoparticles (1:250, 1:500 and 1:1000) and the intensities of the free DNA were not different by visual inspection. In conclusion, gelatin A nanoparticles had higher DNA loading capacity compared with gelatin B nanoparticles. Gelatin A nanoparticles were selected as the gene delivery vector.

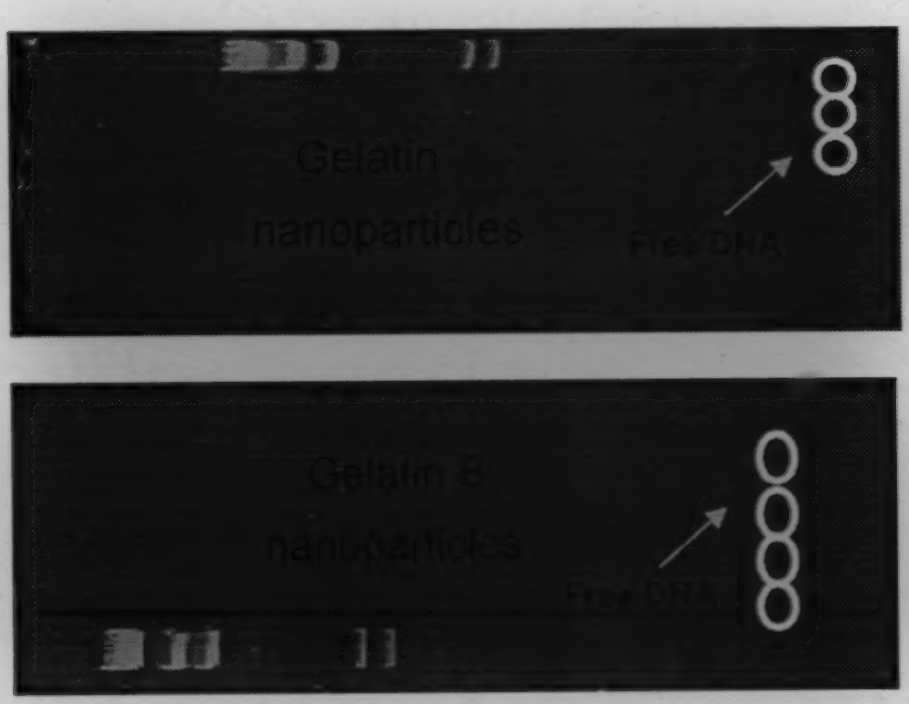

Figure 4.6 Agarose gel electrophoresis of nanoparticles and DNA binding

4.3.2. Discussion

The result of DNA binding studies show the adsorption of DNA onto the surface of gelatin A nanoparticles although their surface charge is negative.

The adsorption of DNA onto gelatin nanoparticles was performed by the incubation of DNA with gelatin nanoparticles in double distilled water. The pH of the water was around 7.4. Although the surface charge of nanoparticles is positive at lower pH (see Figure 4.5), the DNA binding study has to be performed at pH around 7.4, because the transfection experiment will be done in the cell culture medium (the pH of cell culture medium is around 7.4).

Based on the result of zeta potential against pH, gelatin A and B nanoparticles are both negatively charged at pH 7.4. DNA is also highly negatively charged due to the repeating phosphate groups along its backbone. As a result, there are repulsion forces since the DNA molecules and the gelatin nanoparticles both have a net negative charge. But at the same time, hydrogen bonding and van der Waals forces exist and may be responsible for the adsorption of DNA to the surface of the nanoparticle. This means that the attractive forces from hydrogen bonding are stronger compared to the repulsive electrostatic forces. These net attractive forces lead to the adsorption of DNA onto the surface of gelatin A nanoparticles.

The different DNA loading capacity between gelatin A and gelatin B nanoparticles may be explained by the more negative zeta potential of gelatin B nanopatilces compared with gelatin A nanoparticles. This leads to stronger repulsive forces. Therefore, the less negatively charged gelatin A nanoparticles had higher DNA loading capacity compared to gelatin B nanoparticles.

4.4. MTT cytotoxicity assay

After 3 hours of incubation, there was not an obvious of death of cells observed by visual inspection under microscope in COS-1 and 143B cell lines. The different concentrations (0.5-3.0 μg/μL) of naked gelatin A nanoparticle used in the MTT assay did not have any effect on the cell viability. After this, the long time incubation was tested. After the long time incubation (3 days), the nanoparticle showed some cytotoxicity depending on their concentration. The results of the cytotxicity evaluation of naked gelatin nanoparticles using COS-1 and 143B cells (3 day incubation) are illustrated in Figure 4.7.

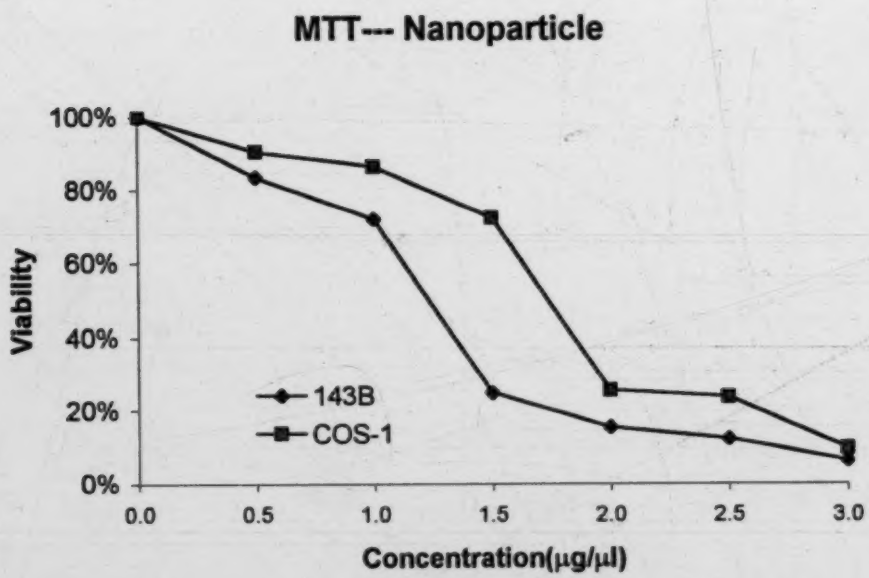

Figure 4.7 Percent of cells surviving following 72h exposure to various concentrations of gelatin A nanoparticles

From the Figure 4.7, we can see that gelatin A nanoparticles have an obvious effect on the cell viability after 3 days exposure. The IC_{50} (the concentration of the test compound that kills 50% of the cells) are around 1.25 μg/μL and 1.75 μg/μL in 143B and COS-1 cells, respectively. In 3.0 μg/μL of gelatin A nanoparticles, the viability of 143B and COS-1 cells was about 10%.

The cytotoxicity of gelatin nanoparticles may come from the crosslinker – glutaraldehyde (GA). Eybl *et al.* found that GA was released from fixed pericard patches even after rinsing. And continuous incubation of bovine aortic endothelial cells with GA > 0.1 mg/L resulted in a significantly inhibited proliferation (Eybl *et al.*, 1989). Huang-Lee *et al.* also reported that cytotoxic level of GA released from crosslinked tendons for at least 6 months. The toxic effect was demonstrated by the death of fibroblasts surrounding crosslinked tendon (Huang-Lee *et al.*, 1990). These results are helpful to explain the cytotoxicity of gelatin nanoparticles.

The toxicity of gelatin nanoparticles has to be determined in animals before clinical trials. Although the gelatin nanopraticles are toxic *in vitro*, they might be not toxic when they are used *in vivo*. Because when the nanoparticles are introduced into animals, the body fluid will dilute the nanoparticles. The defending mechanisms like detoxification function of liver may also help to decrease the toxicity of nanoparticles. The results of liver function test are good clinical parameters for the toxicity of nanoparticles.

4.5. Transfection study

LacZ is a bacterial gene that is used as a reporter gene for detection of trasfection. The LacZ gene encodes for the enzyme β-galactosidase. β-galactosidase catalyzes the hydrolysis of the substrate X-gal (5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside) that results in a blue colored staining of transfected cells (see Figure 4.8 and 4.9). The blue color is easily visualized with a microscope. The percentage of the blue color cells is used as a parameter for the evaluation of the transfection efficiency.

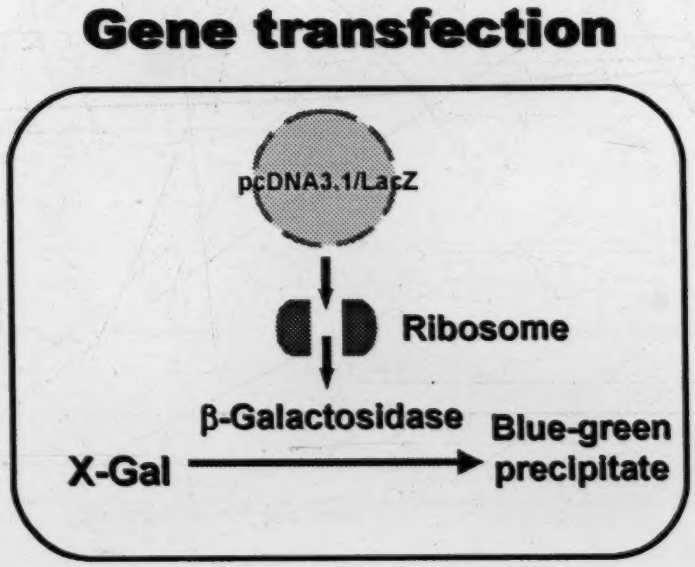

Figure 4.8 Scheme of LacZ gene expression

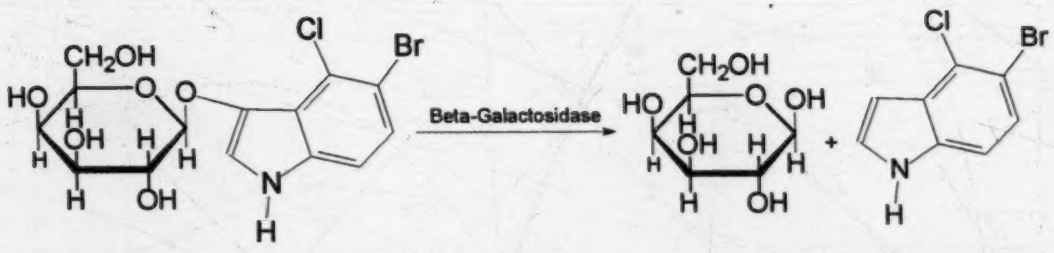

Figure 4.9 Scheme of β-galactosidase catalyzes the hydrolysis of the substrate X-gal

4.5.1. Results

Based on the result from the DNA binding studies, gelatin A nanoparticles were selected as gene delivery vector. Firstly, DNA-nanoparticle complexes with different ratios (1:200, 1:500, 1:1000 and 1:2000) were used for transfection in COS-1 cell line (see Table 4.5). The positive result happened in the ratios of 1:1000 and 1:2000 (DNA: Nanopaticles). This result confirmed the information acquired from the agarose gel electrophoresis: in the ratio of 1:1000 (DNA: Nanopaticles), DNA almost completely attached to the surface of gelatin A nanoparticles. Because the results from the ratios of 1:1000 and 1:2000 (DNA: Nanopaticles) were similar, the ratio of 1:1000 was chosen to test the transfection of gene loaded nanoparticles in different cell lines.

Table 4.5 Transfection results of different ratio (W/W) of DNA and gelatin A nanoparticles in COS-1 cell line

DNA:NP A	1:200	1:500	1:1000	1:2000
Transfection result	-	-	+	+

The concentration of gelatin A nanoparticles in 1:1000 ratio was 3.72 μg/μL. Based on the result of the cytotoxicity study, this concentration had an obvious negative effect on cell viability. But in transfection study, there was not an obvious of death of cells and the concentration of nanoparticles used in gene transfection did not have effect on the cell viability. The exposure time might be the reason. In the cytotoxicity study, the cells were exposured to nanoparticles for 3 days. While in the transfection study, the cells were exposured to nanoparticles for only 3 hours.

COS-1, 143B, Hela and 293 cell lines were used in transfection studies. Figure 4.10 (a) and Figure 4.11 (a) showed the results collected from the positive controls. The LipofectAmine (cationic lipid reagent) mediated transfection yields high transfection efficiencies in a wide variety of eukaryotic cells.

The negative controls did not stain which indicated that the cells could not incorporate the DNA.

In the experimental group, gelatin A nanoparticles transferred the LacZ gene and led to the expression of β-galactosidase in COS-1 and 143B cell lines by the ratio of 1:1000 (DNA: nanoparticles). The transfected cells were seen by blue color when

treated with X-Gal. It was shown in Figure 4.10 (b) and Figure 4.11 (b). There were no positive result in Hela and 293 cell lines.

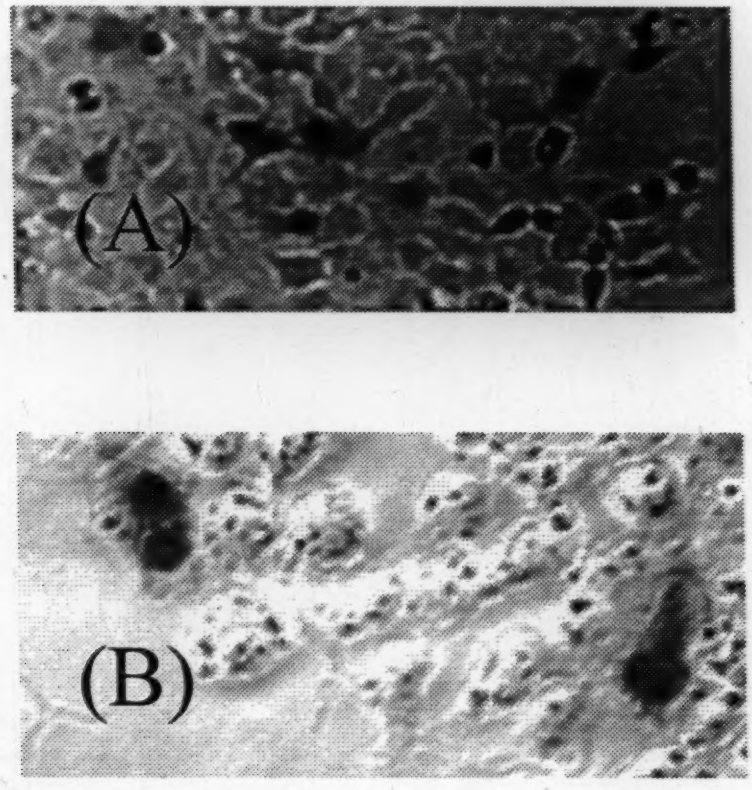

Figure 4.10 Staining of COS-1 cells for β-galactosidase. (A) Positive control, transfected with reagent LipofectAmine, and (B) Experimental group, transfected with gelatin A nanoparticles

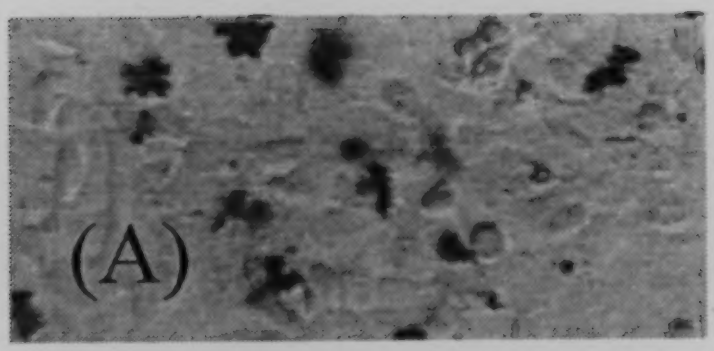

Figure 4.11 Staining of 143B cells for β-galactosidase. (A) Positive control, transfected with reagent LipofectAmine, and (B) Experimental group, transfected with gelatin A nanoparticles

4.5.2. Discussion

Generally transfection can be divided into two classes, stable transfection and transient transfection. In stable transfection, the transfected DNA is integrated into the chromosomal DNA. The added DNA is incorporated into the host genome by recombination. Stable gene expression requires several weeks to perform. In transient expression, the transfected DNA is not integrated into the chromosome. Expression of transfected gene can be detected for 24-96 hours. In a few days, most foreign DNA is diluted by cell division or degraded by nucleases. After one week, the transient expression cannot be detected. In our experiment, expression of transfected gene was detected after 24 hours.

The results of transient transfection studies are summarized in Table 4.6. From these results, we can see that gelatin A nanoparticles are able to deliver LacZ gene to certain cell lines.

Table 4.6 Gelatin A nanoparticles mediated transfection in different cell lines

	COS-1	143B	Hela	293
NP A + DNA	+	+	-	-
Positive Control (LipofectAmine+DNA)	+++			
Negative Control (DNA only)	-			

Gene transfer in eukaryotic cell is a complicated multi-step process. Gelatin nanoparticles may facilitate the uptake in the different steps.

(1). DNA complexation.

Generally, because plasmid DNA is a large, negatively charged molecule up to 1 μm long (Abdallah *et al.*, 1995), and the surface of cell membrane is negative. It is hard to make the plasmid DNA to internalize into cells. The adsorption of plasmid DNA onto nanoparticles may condense the DNA to a smaller size and facilitate the internalization.

(2). Cellular uptake

Cell uptake studies of the gelatin nanoparticles by Kaul and Amiji confirmed that they were internalized by endocytotic pathway and remained stable during the vesicular transport process. Once the nanoparticles were endocytosed, they were able to escape the endosome and found primarily in the cytoplasm around the nuclear membrane (Kaul and Amiji, 2002). The results of this study are very encouraging for the development of a gene delivery system by gelatin nanoparticles.

The result reported by Chavany *et al.* in 1994 supports the idea that nanoparticles can improve the cellular uptake of oligonucleotides. Cellular uptake of oligonucleotides was increased when they were adsorbed onto polyisohexylcyanoacrylate nanoparticles as a result of the capture of nanoparticles by an endocytic/phagocytic pathway (Chavany *et al.*, 1994).

(3). DNA protection

In our project, the LacZ gene was adsorbed onto gelatin nanoparticles. Compared with DNA encapsulated into particles, in this method the release of DNA is easy because desorption is not difficult. And although DNA was not encapsulated into nanoparticles, its adsorption onto the nanoparticles may increase its stability. Similar result can be obtained form research reported by Chavany *et al.* in 1994. They proved that oligonucleotides could be adsorbed on plolyisohexylcyanoacrylate nanoparticles. Oligonucleotides bound to nanoparticles were protected form nuclease attack both in buffer and in cell culture media. Intracellular stability towards nucleolytic degradation was increased in the presence of nanoparticles (Chavany *et al.*, 1994). Fattal *et al.* also found that the oligonucleotides adsorbed onto the surface of polyalkylcyanoacrylate nanoparticles were protected from the nuclease attack in cell culture media and their cellular uptake was increased. The stability of the oligonucleotides was improved *in vivo* when they were adsorbed onto the nanoparticles (Fattal *et al.*, 1998). This improved stability will slow down their clearance from blood, and increase their bioavailability.

In conclusion, gelatin A nanoparticles have the potential to be used as a gene delivery vector, although their transfection efficiency is much lower compared to the commercial cationic compounds. The reason of this low transfection efficiency might due to the interaction of DNA and cell membrane. DNA and cell membrane are both negatively charged, and the adsorption force between the plasmid and the surface of the nanoparticles is weak. When the nanoparticle comes close to the negative cell

membrane, desorption of DNA from the nanoparticles surface may happen before the DNA nanoparticle complex is taken up by cells. This might be the reason of the low transfection rate. Cell uptake studies using 143B cells have shown that nanoparticles are taken up by these cells.

However, the results obtained by this study encourage further studies into the use of nanoparticles as gene delivery vector. Further studies are required to improve the efficiency of transfection.

Chapter 5

Conclusion and Future work

Conclusion

The objective of this project was to investigate gelatin nanoparticles for gene delivery. To achieve this objective, gelatin nanoparticles were prepared. Our systematic investigation of the synthesis parameters showed that controlling the experimental conditions, nanoparticles with defined size ranges and narrow size distribution can be synthesized. Molecular weight, pH at the second desolvation step, type of desolvating agent, amount of glutaraldehyde temperature, and nature of gelatin were the critical parameters for the synthesis of gelatin nanoparticles. The smallest particles were prepared using gelatin B at 40 °C by ethanol as the desolvating agent.

The DNA binding capacity of gelatin A and B nanoparticles was conformed by agarose gel electrophoresis. Gelatin A nanoparticles were selected as vector for gene transfection, because gelatin A nanoparticles showed a higher DNA binding capacity compared with gelatin B nanoparticles. Based on the results, the gelatin A nanoparticles showed the ability to transfect Lac Z gene into COS-1 and 143B cell lines.

Future work

The study reported here is the base of further studies to evaluate the use of gelatin nanoparticles for gene delivery. The results reported in this thesis have raised the following questions that need more investigations.

- The transfection efficiency of nanoparticles needs to be increased. Increasing the transfection efficiency can be tried by decreasing the size of nanoparticles and introducing positive charges to the surface of nanoparticles.
- To increase the transfection efficiency, membrane-active domains may be incorporated into nanoparticles. After internalization by endocytosis, the DNA vectors are enclosed in endosomes or lysosomes. Lysosomal nucleases can degrade the adsorbed DNA. The membrane-active peptides or proteins can help to release the endocytosed DNA into the cytoplasm by lysis of the endosome or lysosome. Overcoming this endosomal barrier will increase the transfection efficiency (Ogris and Wagner, 2002).
- To decrease the cytotoxicity of nanoparticles, natural occurring cross-linking agents like genipin can be used (Liang *et al.*, 2003). In a study performed by Liang *et al.*, genipin was used as a crosslinker. The inflammatory reaction of the tissues implanted with the genipin-crosslinked microspheres was significantly less compared with that implanted with the glutaraldehyde-crosslinked microspheres. Another alternative is thermal stabilization as reported by Weber *et al.* (Weber *et al.*, 2000a).
- Antibodies can be attached to nanoparticles in order to target specific cells.

- The immunogenicity of gelatin nanoparticles can be tested in animal models.
- PEG may be attached to the surface of nanoparticles. This type of surface modification reduces the interaction of the nanoparticles with blood components. It may increase the circulation of nanoparticles in the blood stream to achieve a targeting delivery.

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